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The manual gives revised procedures for Level 1 environmental assessment biological tests, and supersedes the first edition, EPA-600/7-77-043 (NTIS No. PB 268484), published in April 1977. The revised biological procedures complement the Level 1 chemical and physical procedures published in October 1978 as IERL-RTP Procedures Manual: Level 1 Environmental Assessment (Second Edition), EPA-600/7-78-201 (NTIS No. PB 293795). Level 1 is a screening phase that identifies, categorizes, and ranks the pollutant potential of influent and effluent streams from industrial and energy-producing processes. The manual is a guide to sampling and analysis professionals in planning and executing the bioassay portion of a phased environmental source assessment program. The manual gives the goals, strategies, and philosophy of a phased approach to environmental assessment. It introduces collection and pretest handling procedures for environmental samples and the recommended Level 1 biological test protocols used to analyze the samples. Basic quality control procedures are discussed, as are possible bioassay procedures for Levels 2 and 3.

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IERL – RTP PROCEDURES MANUAL: LEVEL 1 ENVIRONMENTAL ASSESSMENT BIOLOGICAL TESTS

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FOREWORD

This bioassay procedures manual has been prepared as a guide for studies to be conducted by the Industrial Environmental Research Laboratory of the Environmental Protection Agency (EPA), Research Triangle Park, North Carolina. To assist in its preparation, a subgroup of the Environmental Assessment Steering Committee was formed. The subcommittee, composed of EPA experts in health and ecological effects, was given the responsibility of recommending specific bioassays. This subcommittee recommended an initial series of tests which were reviewed by the committee as a whole, various other bioassay experts within EPA and others in industry and universities. This manual is a revision of and supersedes the "IERL-RTP Procedures Manual: Level 1 Environmental Assessment, Biological Tests For Pilot Studies," published in April 1977.(EPA-600/7-77-043, NTIS PB 268484).

The bioassay procedures in this manual are designed to complement the chemical and physical procedures of the Level 1 environmental assessment program and to be an integral part of a comprehensive source assessment strategy. The purpose of Level 1 is to obtain preliminary information, identify problem areas, and provide the basis for the ranking of streams for further consideration in the overall environmental assessment.

This manual is written to guide sampling and analysis professionals in planning and executing the bioassay portion of an environmental source assessment program. The recommended biotests for testing the toxicity and mutagenicity of feed and waste streams of industrial processes are described with a brief summary of procedures for collecting and preparing the samples to be tested. A more detailed discussion of the sampling program and the procedures for chemical and physical testing of industrial process feed and waste streams is provided in the companion publication: "IERL-RTP Procedures Manual: Level 1 Environmental Assessment, Second Edition," published in October 1978 (EPA-600/7-78-201, NTIS PB 293795). A more detailed discussion of each bioassay procedure is included in the references cited in each chapter of this manual.

Chapter 1 of this manual defines the goals and strategy employed in Level 1 testing and gives the background and philosophy of the phased approach to environmental assessment.

Chapter 2 discusses the Level 1 sampling activities and pretest-handling procedures that can be used for most industrial complexes. For each sample type, the discussion focuses on the general problem as well as specific problems of preparation needed for sampling, the actual sampling procedures and packaging of samples for shipment.

Chapters 3 through 5 specify the Level 1 health effects, aquatic and terrestrial bioassay schemes. The schemes identify the methods of analyses, anticipated output and level of effort required for implementation and the basic format for presenting the results of the tests.

Chapter 6 describes the data management, including data collection forms and an approach to consolidated toxicity assessment for multitest data for health and ecological effects.

Chapter 7 outlines the recommended quality control and documentation procedures necessary to verify the quality of the assays performed.

Chapter 8 provides a brief discussion of testing beyond that defined as Level 1.

ABSTRACT

This manual presents the revised procedures for Level 1 environmental assessment biological tests. The manual supersedes the first edition published in April 1977 (EPA-600/7-77-043, NTIS PB 268484). The biological procedures in this manual are designed to complement the Level 1 chemical and physical procedures published in IERL-RTP Procedures Manual: Level 1 Environmental Assessment (2 ed.), October 1978, (EPA-600/7-78-201, NTIS PB 293795). Level 1 is a screening phase that identifies, categorizes, and ranks the pollutant potential of influent and effluent streams from industrial and energy-producing processes. The manual is written to guide sampling and analysis professionals in planning and executing the bioassay portion of a phased environmental source assessment program. This manual presents the goals, strategies and philosphy of a phased approach to environmental assessment. It introduces collection and pre-test handling procedures for environmental samples and the recommended Level 1 biological test protocols used to analyze the samples. Basic quality control procedures are discussed, as are possible bioassay procedures for Level 2 and Level 3.

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CHAPTER 1

BACKGROUND AND GENERAL INFORMATION

1.1 INTRODUCTION: DEFINITION OF LEVEL 1

The Industrial Environmental Research Laboratory of the Environmental Protection Agency, Research Triangle Park, North Carolina (IERL-RTP), has developed a three-phased approach to performing an environmental assessment--the testing of feed and waste streams associated with industrial and energy processes in order to define control technology needs. Each phase or level involves distinct sampling and analytical activities. While Level 2 and 3 assessments are briefly described in Chapter 8, this biological procedures manual focuses on Level 1 sampling and bioassays. A companion manual describes the chemical and physical analysis procedures for Level 1 and the details of sampling procedures (1).

Physical and chemical characterization of environmental emissions is critical to the definition of, need for and design of control technology. However, the final objective of the IERL-RTP's environment assessment is the control of industrial emissions to meet environmental goals that limit the release of substances that cause harmful human health or ecological effects. Consequently, the testing of industrial feed and waste streams for biological effects is needed as a complement to the physical and chemical data to ensure that the assessment is comprehensive. Biological testing can provide a direct measure of the toxicity and/or mutagenicity of substances to organisms that chemical analysis cannot. This is especially important when dealing with substances for which there is little available data on toxicity or when assessing complex mixtures where synergisms and antagonisms may alter the toxicity of the individual chemical constituents.

It should be stressed that the results of Level 1 tests are not intended for regulatory actions or recommendations, nor are they to be used as tests of acceptability or non-acceptability of emission release. The three-phased sampling and testing strategy was developed to focus available resources (both manpower and dollars) on industrial emissions which have a high potential for causing measurable health or ecological effects and for providing chemical and biological information on all sources of industrial emissions.

1.2 APPLICATION OF LEVEL 1 TESTING AND ITS INTERPRETATION

The phased approach as it applies to Environmental Assessment requires three separate levels of sampling and analytical effort. The first level provides (1) preliminary environmental assessment data, (2) identification of problem areas and (3) the data needed for the ranking of energy and industrial processes, streams within a process and components within a stream, for further consideration in the overall assessment. Level 2 sampling and analysis is designed to provide additional information that will confirm and expand the data base developed in Level 1. The Level 2 results provide a more detailed characterization of biological effects of the toxic streams, define control technology needs and may define the probable cause of a given problem. Level 3 utilizes appropriate sampling and analysis methodology to monitor the specific problems identified in Level 2 so that the toxic or inhibitory components in a stream can be determined exactly as a function of time and process variation for control device development. Sublethal chronic effects are also monitored in Level 3.

1.2.1 Strategy of a Phased Approach for Level 1 Testing

A phased approach offers potential benefits both in terms of the quality of information that is obtained for a given level of effort and in terms of the costs per unit of information. This approach has been investigated and compared to the more traditional approaches (2) and has been found efficient in both time and funds required for assessment.

Implementation of a phased approach recognizes that it is impossible to accommodate every conceivable condition on the first sampling or analysis effort. There is a possibility that many streams or even the entire installation may not be emitting hazardous substances in quantities of environmental significance. Conversely, certain streams or sites may have such problems that a control technology development program can be initiated in parallel with a Level 2 effort. If either of these situations can be determined by a simplified set of sampling and analysis techniques (Level 1), considerable savings in both time and money will result. When budgetary limitations require sampling only those installations most in need of control technology, a simplified sampling and analysis methodology can usually establish which installations should be given priority.

The three levels are closely linked to the overall environmental assessment effort. Level 1 identifies the questions that must be answered by Level 2, and Level 3 monitors the problems identified in Level 2 to provide information on chronic effects and for control device design and development. The following situation is an example of the relationship of the levels to each other.

Level 1 biological testing results indicated that a small quantity of an effluent has inhibitory effects on algal growth, adverse effects on a specified percentage (EC_{50}) of the population in the Daphnia bioassay and gives a positive microbial mutagenicity test. Level 1 chemical testing indicated that polycyclic organic materials (POM) were present in significant amounts. Considering these results, Level 2 biological sampling and analysis would determine such factors as toxic effect over a long time period, bioaccumulation at low trophic levels (primary producers and consumers) and persistence of toxicity in the receiving waters. Level 2 chemical testing is used to identify and quantify the POM compounds and any other pollutant classes identified at Level 1 as accurately as possible. This combination of biological and chemical testing can identify the exact nature of the toxic substance(s) and determine if a complex biological effect such as synergism, antagonism or bioaccumulation is occurring. Level 3 testing will be used for long-term continuous monitoring. Chemical testing will provide information on seasonal or feedstock variations of the previously identified toxic substance(s). Long-term biological testing will serve as an integrator of such variations. In addition, Level 3 biological testing will identify possible chronic health and ecological effects. The entire data package can then be used in designing the control technology development program for the stream.

1.2.2 Definition of Level 1 Sampling and Analysis

At the initiation of an environmental assessment, little is known about the specific sampling requirements of a source, hence the emphasis is on survey tests. Sampling and analysis at this level are designed to show, within broad general limits, the presence or absence and, where possible, the approximate levels of toxicity associated with a source. The results of this phase are used to establish priorities for additional testing among a series of energy and industrial sources, streams within a given source and components within streams. Level 1 has as its most important function the ranking of specific streams and components for the Level 2 effort.

1.2.3 Goals of Level 2 Sampling and Analysis

The Level 2 sampling and analysis goal is to provide definitive data required in the environmental assessment of a source. Consequently, the goal of Level 2 sampling and analysis is obtaining statistically representative samples, expanding information on the nature of the biological response and finally, where possible and when necessary, identifying and quantifying the toxic substance(s). Level 2 analyses are the most critical of all three levels because they must provide confirmation of the results obtained in Level 1 and give an accurate characterization of the potential of the source to cause adverse environmental effects.

Level 2 must provide sufficient detailed information on the problems delineated by Level 1 so that control stream priorities, total environmental damage and an initial estimate of process/control system regions of overlap can be established.

1.2.4 Goals of Level 3 Sampling and Analysis

Level 3 testing involves long-term monitoring of components specific to the stream of concern. The sampling and analysis are directed towards the integration of effects over time to account for seasonal or feedstock variations. These efforts are also geared to assess the chronic health and ecological effects of the stream components.

1.3 MULTIMEDIA SAMPLING PROCEDURES

The Level 1 procedures described in the IERL-RTP sampling and chemical analysis manual (1) can be utilized to acquire process effluent and feedstock samples. The Level 1 environmental assessment program, at a minimum, must acquire a sample of each process feedstock and effluent stream, and of fugitive air/water emissions. The feed stream data are necessary to establish a baseline for comparison. The effluent stream sampling program is required to determine the mass emissions rate and the environmental impact which will result.

1.3.1 Classification of Streams for Sampling Purposes

Comprehensive assessments are organized around the five general types of samples found in industrial and energy-producing processes rather than around the analytical procedures that are required to collect the samples. This facilitates the complex task of organizing the manpower and equipment necessary for successful field sampling. The Level 1 chemical and physical manual (1) should be consulted before sampling is undertaken to avoid confusion in classifying streams or stream samples.

The five sample types are:

(1) Gas/Vapor (Non-particulate laden) - These include samples from process streams, vents and effluents. Samples contain inorganic and organic gaseous components.

(2) Gaseous Streams (Particulate or aerosol laden) - This involves sampling contained air or gas streams such as in ducts or stacks. Samples include particulates and higher molecular weight organics with boiling points greater than 100°C.

(3) Liquid/Slurry Streams - Liquid streams are defined as those containing less than 5 percent solids. Slurry streams are defined as those containing greater than 5 percent solids. Liquid or slurry streams are classified as aqueous or nonaqueous. A stream sample that contains more than 0.2 percent organics is considered nonaqueous.

(4) Solids - These include a broad range of material sizes from large lumps to powders and dusts, as well as nonflowing wet pastes. Nonflowing wet pastes may be formed either by wetting solids with aqueous or nonaqueous liquids or may be highly viscous liquids such as some tars or oils. The distinction between solids and slurries can become blurred.

(5) Fugitive Emissions - The characteristic of this general sample type is that the emissions are transmitted to the environment without first passing through some stack, duct, pipe or channel designed to direct or control their flow. The sample may be in any of the above physical forms and may result from non-ducted gaseous, particulate or liquid emissions from the overall plant or process units.

A flow diagram which shows the overall relationship of the samples to the Level 1 analysis scheme is presented in Figure 1.1.

1.3.2 Phased Approach Sampling Point Selection Criteria

The selection of sampling points, where phased sampling techniques are employed, relies on the concept previously stated: that Level 1 sampling is oriented towards obtaining data with relaxed accuracy requirements for determination of the pollution potential of a source, whereas Level 2 sampling is intended to acquire more accurate data necessary for a definitive environmental assessment. The recommendations in this manual are restricted to Level 1 sampling and analysis. Stream parameters such as flow rates, temperature, pressure and other physical characteristics will be obtained on both levels within the objectives of a given level of sampling.

1.4 DATA REQUIREMENTS AND PRETEST PLANNING

Prior to the actual sampling and analysis, the data needs must be established and used to identify analysis requirements and sampling problems. The following paragraphs present a summary of data requirements and planning which must be applied (1-3). Specific recommendations associated with each of the process streams are discussed in the appropriate chapters of this manual.

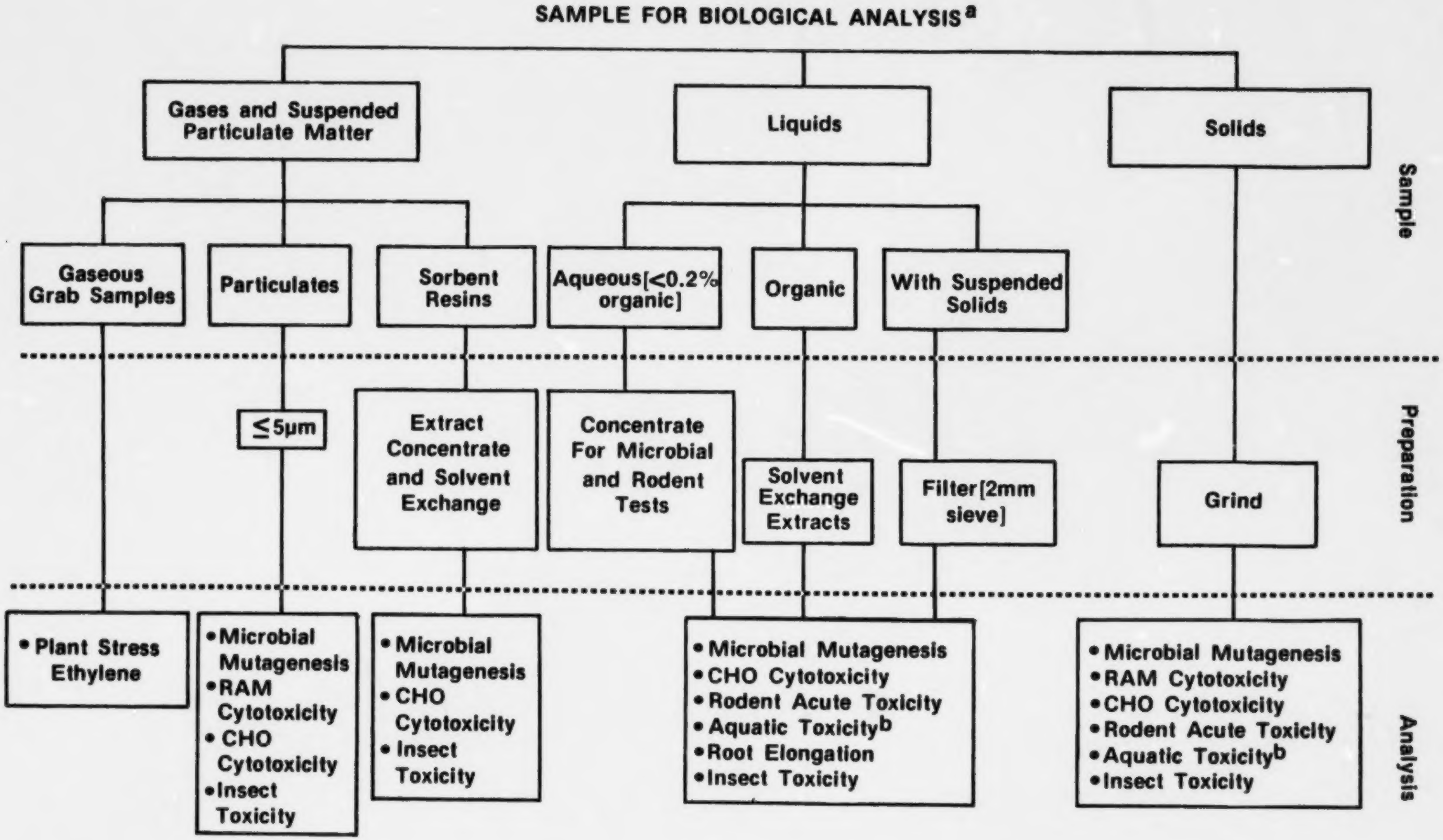
1.4.1 Process Data Needs

Before traveling to a plant for a pretest site survey, it is necessary to become familiar with the chemistry and operational characteristics of the operations as well as any pollution control processes. The detailed process data necessary for the sampling and analysis effort, as well as the overall environmental assessment, are described in the sampling and chemical companion to this manual (1):

The data collected must be consistent with the overall Level 1 objectives. Thus, the minimum amount of data for a given stream is flow rate per unit time at a given temperature and pressure. Additional data that may be necessary are average flow per unit time, the effect of process variations on stream flow and composition, and normal variations in flow and compositions with variations in process cycling. It is expected that professional sampling and analysis personnel, in conjunction with the EPA Project Officer, Industrial Environmental Research Laboratory, Research Triangle Park (PMB-IERL-RTP), will select the appropriate data requirements for a given industry.

1.4.2 Pretest Site Survey

After establishing the necessary process data needs and selecting a tentative set of sampling points, a pretest site survey should be performed. The pretest site survey must include provision for identification of streams for bioassay sample collection. Since the sample requirements


SAMPLE FOR BIOLOGICAL ANALYSIS[a]
Sample
Preparation
Analysis
Gases and Suspended Particulate Matter
Liquids
Solids
Gaseous Grab Samples
Particulates
Sorbent Resins
Aqueous[<0.2% organic]
Organic
With Suspended Solids
≤5μm
Extract Concentrate and Solvent Exchange
Concentrate For Microbial and Rodent Tests
Solvent Exchange Extracts
Filter[2mm sieve]
Grind
• Plant Stress Ethylene
• Microbial Mutagenesis • RAM Cytotoxicity • CHO Cytotoxicity • Insect Toxicity
• Microbial Mutagenesis • CHO Cytotoxicity • Insect Toxicity
• Microbial Mutagenesis • CHO Cytotoxicity • Rodent Acute Toxicity • Aquatic Toxicity[b] • Root Elongation • Insect Toxicity
• Microbial Mutagenesis • RAM Cytotoxicity • CHO Cytotoxicity • Rodent Acute Toxicity • Aquatic Toxicity[b] • Insect Toxicity

[a] Consult Table 2.3 for Recommended Test Sample/Bioassay Capabilities and Table 2.4 for Test Sample Preparation.

[b] Aquatic Tests Include Freshwater or Marine Fish, Invertebrate and Algal Tests.

Figure 1.1 BIOLOGICAL ANALYSIS OVERVIEW

for bioassay testing are larger than requirements for chemical analysis, the presite survey must be sufficiently detailed to allow definition of the correct process stream, the proper location and sampling methodology prior to the arrival of the field sampling team. This presite survey may or may not recommend the same sampling methods for chemical and biological analysis, however, both types of samples should represent the same process stream over approximately the same time period.

CHAPTER 2

SAMPLE COLLECTION, STORAGE, IDENTIFICATION, AND PRETEST HANDLING

2.1 INTRODUCTION (1,3,4)

Level 1 sampling strategy presumes that all streams leaving the process will be sampled unless empirical data equivalent to Level 1 already exist. Further, Level 1 sampling is not based on *a priori* judgements as to the composition of streams. It is presumed that prior knowledge about the source is, at best, incomplete. Predictions and extrapolations of existing data should be used only as a check on Level 1 assessments and not as a replacement for it (3).

Level 1 sampling programs make maximum use of existing sampling and stream access sites. While care must be exercised to limit sample bias, the commonly applied concepts of multiple point, isokinetic or flow proportional sampling are not rigidly adhered to. A series of discrete samples are taken, when appropriate, and are combined proportional to stream flow to produce a single "average" for analysis. Alternatively, a single sample of each stream is collected under average process operating conditions.

This chapter briefly discusses the general methodology for obtaining gaseous, particulate, liquid and solid feedstock and waste-stream samples for the biological analyses. An overview of the sampling procedures is presented in Table 2.1. A more detailed description is included in the IERL-RTP Procedures Manual: Level 1 Environmental Assessment (Second Edition) (1).

The types of samples obtained from the procedures outlined in Table 2.1 are usually mixtures and present problems for biological test systems that have been developed and validated primarily with pure chemicals. Table 2.2 summarizes the characteristics of samples collected from various sources.

All Level 1 bioassays are not suitable for the entire range of sample types obtained from industrial sources. Certain tests, for example, provide reliable results with solid samples that are soluble in organic solvent carriers, but the same test is not reliable if used to evaluate a gas or slurry. In other situations, the amount of sample required for applicable bioassays is too large to permit the tests to be performed on the available sample (such as with SASS samples).

Suitability of test systems for specific samples must be judged on an individual basis but, as shown in Table 2.3, some generalizations and recommendations can be made with respect to bioassay/test sample compatability.

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TABLE 2.1 SUMMARY OF SAMPLING METHODS

Source Type	Number of Samples	General Method	General Follow-Up
Gas and Vapor (Non-particulate Laden)	1	· High pressure line · Grab purge · Evacuated grab	A glass-bulb sample container is used for chemical and some bioassay analysis subsamples. Teflon or Tedlar bags are used to hold and transport larger samples for bioassays.
Gaseous Streams Containing Particulates	1 process cycle or 5 hours continuous sample	SASS[a]	Particulates (10, 10-3, 3-1 μm) are collected in cyclones and on filters (<1μm). Gaseous materials are collected in XAD-2 cartridges. Ship particulate samples and XAD-2 samples in amber glass.
Liquid/Slurry	1	· Heat exchange · Tap · Dipper · Automatic	Samples maintained at 4°C in closed glass containers until subjected to analysis.
Solid	1	· Manual grab · Boring or auger techniques	Samples maintained in sealed amber glass
Fugitive Emissions (Air or Water)	1	Air: high volume sampler equipment with a 3.5 μm filter and an XAD-2 cartridge, evacuated grab sampler, or SASS. Water: plug collectors	Airborne particulates and XAD-2 column samples handled as described above. Water samples maintained at 4°C in closed containers until subjected to analysis.

[a] SASS - Source Assessment Sampling System, Aerotherm Corporation, 485 Clyde Avenue, Mountain View, CA, 94042.

TABLE 2.2 LEVEL 1 BIOASSAY SAMPLE CHARACTERISTICS

Source	Sample	Description	Characteristics
Air			
Gas/Vapor (Non-particulate laden)	Grab	Gas	Organic, inorganic or both. Sample limited by storage capacity.
Gaseous Streams (particulate/aerosol laden)	SASS Cyclone (10 µm + 3 µm)	Solids > 3 µm	May be inorganic, organic or both. SASS samples may have limited size.
	SASS - Cyclone (1 µm)	Solids 1-3 µm	Same as above.
	SASS - Filter	Solids < 1 µm	On fiberglass mat. Combine with SASS 1-3 µm if possible.
	SASS XAD-2 Resin	XAD-2 extract	Organics in dichloromethane. Requires solvent exchange before bioassay.
Process Fugitive Emissions	High-Volume Sampler	Solids	Organic, inorganic or both.
Fugitive Gases	High-Volume Sampler	XAD-2 extract	Same as SASS XAD-2 Resin
	Grab	Gas	Same as Grab above
Liquids			
All Sources	Grab or Composite	Untreated	Aqueous, nonaqueous or organic. Solution, suspension or slurry. Unlimited sample except for fugitive run-off.
Solids			
Piles, Conveyors, Bins, etc.	Grab or Composite	Untreated solids	Coal, ash, residues, products; organic and inorganic; unlimited sample.

TABLE 2.3 RECOMMENDED TEST SAMPLE/BIOASSAY COMPATABILITIES

Sample Type	Health Effects Bioassays[a]				Ecological Effects Bioassays[a]			
					Aquatic Tests[b]	Terrestrial Tests		
	Ames	RAM	CHO	WAT		PSE	RE	IT
1. Gas/Vapor (Non-particulate)	B[c]	NC	NC	NC	NC	R	NC	B
2. Liquids (<5% Solids)								
A. Aqueous	R	A	R	R	R	B	R	R
B. Nonaqueous[d]	R	A	R	A	A	B	A	R
3. Solids and Slurries (>5% Solids)								
A. Soluble	R	A	R	R	R	B	R	R
B. Insoluble	R	R	A	R	R	B	R	R
C. SASS particulates	R	R	A	NC	NC	NC	NC	A

[a] Standard test abbreviations are as follows:

Ames: Ames Salmonella/microsome mutagenesis assay.
RAM: Rabbit alveolar macrophage cytotoxicity assay.
CHO: Rodent cell clonal toxicity assay.
WAT: Acute *in vivo* test in rodents (whole animal test).
PSE: Plant stress ethylene test.
RE: Root elongation test.
IT: Insect toxicity assay.

[b] Aquatic tests include marine or freshwater fish, invertebrate and algal bioassays.

[c] Identification of compatibility abbreviations:

R: Recommended for Level 1 environmental assessment testing.
NC: Sample not compatible with test methodology.
A: Compatible with bioassay with no modifications to protocol. Not recommended for routine Level 1 testing, but may provide additional information.
B: Compatible with bioassay with modification to protocol. Not recommended for Level 1 testing.

[d] Nonaqueous liquids include samples with greater than 0.2 percent organics, solvent exchange samples and sorbent resin extracts. Extracts must be solvent exchanged to dimethylsulfoxide (DMSO) for testing.

2.2 PROCEDURES FOR SAMPLE IDENTIFICATION, PACKAGING, AND TRANSPORT

In order to evaluate samples properly, information regarding the collection, processing and shipping of these samples must be transmitted by the sampling contractor with the samples to the bioassay contractor.

The forms provided here are proposed for use by the field sampling Manager and the EPA Project Officer to transmit the necessary information. The first form, "Level 1 Bioassay Sample Collection Form," (Figure 2.1) details the manner in which the samples are collected and processed before shipment to the biological testing laboratory. The second form, "Level 1 Sample Processing Form" (Figure 2.2), defines the types of bioassays to be performed on the sample. It is completed by the Project Officer responsible for the environmental assessment. Copies of the sample collection forms should be attached to all final reports.

Upon receipt of each test substance in the laboratory performing the bioassays, the following information should be recorded in a permanent copy and maintained on file for reference purposes:

(a) Date of Sample Receipt
(b) Sponsor's Name
(c) Sample Identification
(d) Quantity and Condition of Sample Received
(e) Physical Description of the Sample
(f) Storage Conditions and Location
(g) Sample Disposition and Disposal

These records are for internal quality control purposes and need not be attached to the report.

An acceptable system of sample coding is listed below. This system will permit more rapid identification of samples and yet maintain a high degree of uniformity between chemical and bioassay sample codes.

1C	1-3μm cyclone catch
3C	3-10 μm cyclone catch
10C	> 10 μm cyclone catch
PF-a	Particulate filter(s)
PR	CH_2Cl_2/Methanol probe and cyclone rinse
MR	CH_2Cl_2 organic module rinse
XR	XAD-2 resin
XRB	XAD-2 resin blank
CD-O	Neat condensate
CD-LE	CH_2Cl_2 extract of condensate
CD-AE	Acidified, extracted condensate
HM	HNO_3 module rinse
HMB	HNO_3 blank
HI	First (H_2O_2) impinger - Special handling
HIB	First (H_2O_2) impinger blank - Special handling
AI	2nd and 3rd (APS) impinger composite
AI-1B	2nd (first APS) impinger blank
AI-2B	3rd (second APS) impinger
MCB	CH_2Cl_2/blank
MMB	CH_2Cl_2/Methanol blank
FF	Liquid (oil) fuel feed
CF	Solid (coal) fuel feed
FA	Fly ash
BA	Bottom ash

FIGURE 2.1 LEVEL 1 BIOASSAY SAMPLE COLLECTION FORM

I. SAMPLE INFORMATION

1. Sample No. ____________________ Collection Date ____________
2. Sampling Site ____
3. Field Sampling Manager (on-site) ____________________________
4. Contractor ____________________ Contract No. ______________
5. EPA Project Officer ____________ Program Name ______________
6. Source Sampled ____
7. Discharge Rate of Source (Volume/Time) ____________________
8. Quantity Sampled/Units _____________________________________
9. Sample Description (liquid, slurry, solid, extract, appearance, etc)

   ____

10. Other Information as Applicable

    Collection temp. _____________ Sampling location _____________
    pH __________________________ Sampling technique ____________
    Other ____

II. HANDLING & SHIPPING

1. Describe Sample Treatment Prior to Shipping (e.g., transfers, extractants, stored undiluted, grinding, solvents used) __________

   ____

2. Field Storage and Shipping Conditions

Container	Temperature	Light
☐ Amber Glass	☐ Ambient	☐ Shield from light
☐ Polyethylene Bottle	☐ Refrigerate (0 to 4°C)	
☐ Coated Bag or Bottle	☐ Freeze (-20°C)	
☐ Teflon or Tedlar Bags	☐ Dry Ice	
☐ Other ____________		

3. Approximate Time in Storage and Time in Shipping ______________
4. Sample Shipped to ____
5. Mode and Carrier for Shipping ______________________________
6. Comments ____

   ____

(This form should be completed by the on-site sampling manager and accompany each sample. A copy should be forwarded to EPA project officer and should be attached to the Final Report). (Information necessary for bioassay contractor to perform tests and additional information on source can be found in engineering report.)

FIGURE 2.2 LEVEL 1 BIOASSAY SAMPLE PROCESSING FORM

I. SAMPLE IDENTIFICATION

Sample No. ________________ Collection Date ________________
Bioassay Contractor ____________ Program Manager ________________
EPA Project Officer ________________________________
Brief Sample Name ________________________________

II. SAMPLE TYPE AND LAB PROCESSING

Basic Type	Subtype	Processing
Solid	☐ Solid Granular	☐ Grind, ☐ <5 μm or ☐ <3/8 in.
	☐ Slurry, > 5% Solids	☐ Extract Particulates with Organic Solvent
	☐ Particulates from Filter	
	☐ Filter and Particulates	☐ Remove Particulate from Filter
		☐ Prepare Water Leachate
Liquid	☐ Suspensions, < 5% Solids	☐ Concentrate
	☐ Aqueous	☐ Solvent Exchange
	☐ Nonaqueous	☐ Evaporate to Dryness
	☐ Extract	☐ Filter
	☐ Condensate	☐ Test as is
	☐ Other ____________	☐ Other ____________
Gas	☐ Pressure Collection	
	☐ Vacuum Collection	

III. BIOASSAYS REQUESTED AND QUANTITY REQUIRED

Health Effects	Solid	Aqueous Liquid	Nonaqueous Liquid
☐ Mutagenesis (Ames)	2.0 g	5 ml	5 ml
☐ Macrophage toxicity (RAM)	0.1 g	45 ml	2 ml
☐ Rodent Cell Clonal toxicity (CHO)	0.1 g	45 ml	2 ml
☐ Mice *in vivo* toxicity (WAT)	10.0 g	50 ml	20 ml
Aquatic Ecological Effects			
☐ Freshwater fish toxicity	10 kg	40 L	1 L
☐ Freshwater invertebrate	0.5 kg	2 L	0.2 L
☐ Freshwater algae	0.25 kg	1 L	0.1 L
☐ Marine fish toxicity	10 kg	40 L	1 L
☐ Marine invertebrate	2 kg	8 L	0.8 L
☐ Marine algae	0.25 kg	1 L	0.1 L
Terrestrial Ecological Effects			
☐ Plant stress ethylene (PSE) (1,365 liters gas)	---	---	---
☐ Plant root elongation (RE)	2.5 kg	10 L	---
☐ Insect toxicity (IT)	0.5 g	20 ml	10 ml
☐ Other tests (explain on back)			

IV. RECEIPT OF SAMPLE

Date of Receipt ______________ Received by ______________

This form is completed by the EPA project officer and transmitted to the bioassay contractor. The amount of nonaqueous liquid required for aquatic testing is dependent upon the water solubility of the liquid sample.

2.3 SAMPLE PREPARATION PRIOR TO BIOASSAY TESTING

Level 1 environmental sampling procedures provide samples which represent the "average" composition of solid, liquid and gaseous streams of individual processes. Biological testing of these Level 1 samples is generally limited to testing of the whole sample which is consistent with the survey nature of this environmental assessment program. The testing of fractionated samples or specific components of a given sample involves a degree of specificity more appropriate to Levels 2 and 3 testing.

Pretest sample preparation should be limited only to those procedures required to process the sample to a form compatible with biological testing. Pretest processing for specific sample types should be standardized and applied as uniformly as possible across all Level 1 tests as shown in Table 2.4. Data evaluation may be skewed if test materials are not subjected to identical pretest processing in all bioassays. The final ranking of a process stream for potential toxicity is based on a composite of bioassay results. If some of the bioassay results are derived from extracted or concentrated samples and other test results are based on whole, unprocessed samples, the composite ranking may be skewed toward greater toxicity. When samples are processed prior to testing, the results of the tests should be adjusted to reflect the changes in concentration introduced during the processing procedure. This chapter includes a brief description of how samples should be handled in preparation for biological analysis.

2.3.1 Solid Material Grinding and Particle Sizing

Insoluble solid samples should be ground to particles of 5 μm or less in size. Grinding should be accomplished in a way which does not heat the sample. Manual methods such as mortar and pestle or automatic methods such as cryoscopic impact grinding* may be used. Cryoscopic impact grinding is accomplished at liquid nitrogen temperatures. Approximately 100 to 250 mg of the sample are placed in the impaction unit dry or along with a small amount of a suitable vehicle, such as absolute ethanol. The sample is recovered from the grinder in a larger volume of vehicle.

The suspension is then run through a filter system utilizing 25 μm nylon prefilter and a 5 μm nylon final filter.† The 5 μm particle criterion in the resulting filtrate can then be verified by light microscopy. Samples can then be evaporated to dryness, weighed and resuspended in the appropriate medium for the biological tests.

2.3.2 Organic Extraction of Particulates

In addition to testing solids and particulates directly, individual projects or certain bioassays such as the Ames test may require the preparation and testing of organic extracts of these sample types. Solid materials including SASS cyclone probe and filter particulate, ash, and other samples collected from high-temperature process streams may be extracted for 24 hours with dichloromethane in a Soxhlet apparatus (1).

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*For example, Spex Freezer/Mill, Spex Industries, Inc., Metuchen, NJ 08840.
†For example, Tetko, Inc., 422 Saw Mill River Road, Elmsford, NY 10523.

TABLE 2.4 PRETEST SAMPLE PREPARATION

	Health Effects Tests[a]				Ecological Effects Tests[a]			
					Aquatic	Terrestrial Tests		
Sample Type	Ames	RAM	CHO	WAT	Tests[b]	PSE	RE	IT
1. Gas/Vapor (Non-particulate)	--[c]	--	--	--	--	WS	--	--
2. Liquids (<5% Solids)								
A. Aqueous	CX	(WS)	WS	CL	WS	--	WS	WS
B. Nonaqueous[d]	SE	(SE)	SE	(SE)	(SE)	--	(SE)	SE
3. Solids and Slurries (>5% Solids)								
A. Soluble	WS	(WS)	WS	WS	WS	--	(WS)	WS
B. Insoluble	EX	GR	(GR)	WS (LE)	LE	--	(LE)	EX
C. SASS particulates	FE,EX	FE	(FE)	--	--	--	--	(FE,EX)

[a] Standard test abbreviations are explained in Table 2.3.

[b] Aquatic tests include marine or freshwater fish, invertebrate and algal bioassays.

[c] Identification of sample preparation abbreviations:
CL: Concentrate 4- to 10-fold by lyophilization (Section 2.3.8)
CX: Test neat sample in minimum Ames test. If negative, concentrate up to 1000-fold and solvent exchange to DMSO (Sections 2.3.4 and 2.3.6).
EX: Test whole sample in standard test. If negative, at the direction of the Project Officer, extract organics from particulates, solvent exchange to DMSO (Section 2.3.2) and retest.
FE: Test SASS particulates as supplied, for 1 μm or less fraction, remove particulate from filter (Secton 2.3.7).
GR: Grind to 5 μm or less (Section 2.3.1).
LE: Test aqueous leachate of insoluble solids (Section 2.3.9).
SE: Test nonaqueous liquids without preparation, extract sorbent resins and solvent exchange to DMSO (Sections 2.3.3 and 2.3.6).
WS: Test gas or aqueous liquid samples as supplied and soluble solids as solutions.
( ): Sample compatible with test, but not required for Level 1 testing (see Table 2.3).
--: Sample not compatible with test or test requires modification, not required for Level 1 (see Table 2.3).

[d] Nonaqueous liquids include samples with greater than 0.2 percent organics, solvent exchange samples and sorbent resin extracts.

After cooling, the nonvolatile organic content of the extract is determined by gravimetric (GRAV) analysis. An aliquot, not to exceed 10 percent of the sample, is processed by the GRAV method as described in Section 2.3.5 of this manual. The remainder of the sample is evaporated to dryness under nitrogen at $\leq$40°C. The sample is then treated as a nonvolatile organic solid, and redissolved or suspended in DMSO. The objective is to achieve a final concentration of 15 mg (minimum) to 100 mg (preferred) of organics per milliliter of dimethylsulfoxide (DMSO) in a sufficient volume of sample for testing.

Extracts of solid or particulate samples collected in a manner that prevents volatilization of moderately boiling organics (bp 100° to 300°C) are analyzed for total organics, concentrated and solvent exchanged by the procedures used to process SASS sorbent extracts (Sections 2.3.5 and 2.3.6).

2.3.3 Preparation of Sorbent Resin Extracts

The XAD-2 sorbent is extracted for 24 hours in a large Soxhlet extraction apparatus with dichloromethane as described in IERL-RTP Procedures Manual: Level 1 Environmental Assessment (Second Edition) (1). The boiling solvent level is maintained throughout extraction. An aliquot of the sample is assayed for total organic content by the methods discussed in Section 2.3.5. The extract must then undergo concentration and solvent exchange with, preferably, DMSO before incorporation into the microbial mutagenesis, cellular toxicity or insect toxicity tests (see Section 2.3.6). Care must be taken not to bring the resin extract to dryness at any time during concentration or solvent exchange.

2.3.4 Concentration of Organic Material in Aqueous Environmental Samples

Introduction. Organic separation and concentration of water samples for mutagenesis testing for Level 1 is accomplished using XAD-2 and XE-347 porous resins. Both resins are available from Rohm and Haas Co., Philadelphia, PA. After extensive review and experimental work on sorbents (5), it is apparent that no single resin can be used to sample adequately the wide range of chemical classes present in aqueous environmental samples. XAD-2 has high affinity for non-polar species but fairly low affinity for polar compounds. The best resins for adsorption of polar species are the Amber-sorb XE-340 series, with XE-347 exceeding the volumetric capacity of the others. Using this information, a two-stage sampling cartridge is described in this section. Samples entering the cartridge would encounter the XAD-2 first where polar organic material is adsorbed. Then, any polar materials breaking through the XAD-2 would be adsorbed by the XE-347 stage.

The sequential XAD-2/XE-347 sorbent cartridge is recommended for general and compound-specific sampling of organics from water at levels of 10-100 ppm and below. If an aqueous stream of unknown organic loading contains more than 100 parts-per-million of organics, breakthrough may occur before a 10 liter total sample is collected. Also, if more than 1 g of organic material is recovered from the sampling cartridge recommended here, breakthrough should be suspected.

Column Construction. A plan for a full-scale field portable sampling cartridge appears in Figure 2.3. Any system composed of glass, Teflon and/or stainless steel which meets the minimum size requirement may be used. The rationale for the choice of parameters listed here is presented in reference 5.

It has been demonstrated that linear velocities of eluent greater than 4.0 cm/min produce undesirable reduction in the volumetric capacity of the column.

The minimum length of the resin bed was determined by the retention characteristics of the resins used. The column design described here has been shown capable of collecting compounds of interest from water at 95 percent efficiency.

It is generally accepted that an adsorbent bed should have a length-to-diameter ratio considerably greater than one to avoid channeling and back-eddy effects. The cartridge in Figure 2.3 was thus somewhat "over designed" and would be sufficient for collection of maximum of 30 liters, at the specified 42 ml/min flow rate. However, recoveries from the cartridge should not be significantly reduced by using the larger quantity of resin so long as the desorption solvent flow is in a counter direction to the original sample flow, or the resins are separated and extracted separately.

Alternate column sizes and corresponding flow rates are listed in Table 2.5. These sizes meet the minimum requirements for diameter, flow rate and volumetric capacity described above. Table 2.5 is provided to facilitate construction of the proper sampling cartridge from units available from commercial suppliers.*

Preparation of Resins. Resins are cleaned in batches. To remove fines and preservatives, the resins are first washed with deionized water. The remaining material is transferred to a Soxhlet apparatus, and consecutively extracted with three solvents: distilled, deionized water (eight hours), methanol (24 hours) and methylene chloride (24 hours). After extraction, the resins are removed from the Soxhlet apparatus and stored in acid-washed, amber glass bottles under methanol.

Preparation. Packing the trap with the resin beads is easiest when vacuum is applied to the bottom (by aspirator). The cleaned resin beads are loaded with a metal spoon and packed by drawing organic free water through the bed. The XAD-2 resin is loaded first into the bottom stage; then a separating ring is dropped in, followed by the XE-347 resin. Finally, the top is secured and both end caps tightened.

Field and Laboratory Sampling. Aqueous samples are to be concentrated 1000-fold by this procedure. The final requested volume for Ames testing is 5 ml, so 5 liters of aqueous sample are passed through the column by

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*For example, Bio-Rad Laboratories, 2200 Wright Avenue, Richmond, CA 94804.

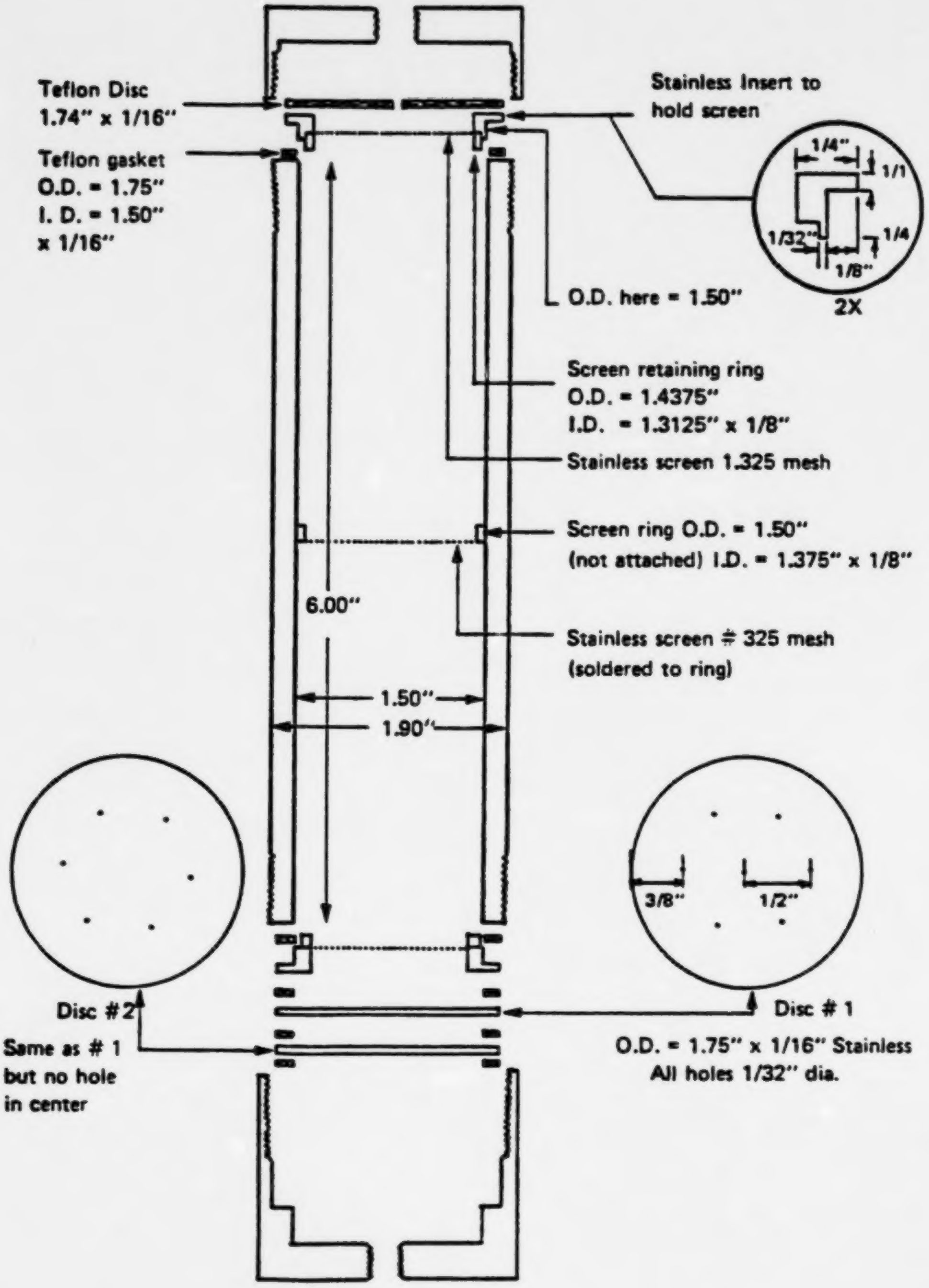
Teflon Disc
1.74" x 1/16"
Teflon gasket
O.D. = 1.75"
I. D. = 1.50"
x 1/16"
Stainless Insert to
hold screen
1/4"
1/1
1/32"
1/4
1/8"
2X
O.D. here = 1.50"
Screen retaining ring
O.D. = 1.4375"
I.D. = 1.3125" x 1/8"
Stainless screen 1.325 mesh
Screen ring O.D. = 1.50"
(not attached) I.D. = 1.375" x 1/8"
6.00"
Stainless screen # 325 mesh
(soldered to ring)
1.50"
1.90"
3/8"
1/2"
Disc #2
Same as # 1
but no hole
in center
Disc # 1
O.D. = 1.75" x 1/16" Stainless
All holes 1/32" dia.

Figure 2.3 FIELD PORTABLE SAMPLING CARTRIDGE [to scale]^5

TABLE 2.5 MINIMUM REQUIREMENTS FOR SAMPLING CARTRIDGES

Column ID cm	Length[a] cm	Maximum Flow Rate ml/min	Time in hrs. Sample 10 liters
1.5	40	7	20
2.0	20	12	13
2.5	13	20	8.5
5	10[b]	42	4

[a] Column length includes provision for both XAD-2 and XE-347. Half-length separate columns of each resin may be connected in series to satisfy this length. Longer column lengths are acceptable, however, an unused cartridge containing XAD-2 and XE-347 should be extracted and subject to bioassay as a solvent-method blank.

[b] Ten (10) cm is the minimum length of the cartridge based on a 2:1 length-to-width ratio.

gravity feed or by using a laboratory metering pump*. All delivery lines should be Teflon or stainless steel connected by swagelock fittings. The first component the sample encounters as it enters the cartridge is a cavity which is filled with glass wool. The purpose of this is to remove any particulate material from the sample stream before it reaches the resin.

Note that the direction of flow is up (against gravity). This is done to minimize problems caused by bubbles or channeling as a result of the action of gravity on the system.

Sample Recovery. The cartridge is loaded in a manner that keeps the two sequential resin beds separate, so that the extraction and analyses of the adsorbed compounds can be made separately. The contents of each half of the cartridge are placed in separate Pyrex Soxhlet thimbles which have been previously cleaned. To remove as much water as possible, these thimbles are attached to suction flasks and aspiration vacuum is applied, drawing most of the water through. After 15-30 seconds of air-drying, the thimbles are placed in Soxhlet extractors. Samples are extracted for 24 hours. The resultant organic samples from the XAD-2 and the XE-347 are combined and are dried using anhydrous sodium sulfate which was cleaned and prepared as described in reference (1). Alternatively the resins may be extracted *in situ* using a continuous extraction apparatus (5). *In situ* extraction must be done in a way that back-flushes the methylene chloride solvent through the XE-347 first, through the XAD-2 second and finally out into the receiving flask. The total organic content of the extract is analyzed according to the methods in Section 2.3.5. Concentration of the organic sample to 5 ml and solvent exchange to DMSO is performed as described in Section 2.3.6 of this manual.

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*For example, Fluid Metering, Inc., Oyster Bay, NY 11771.

2.3.5 Analysis of Extracts and Organic Liquids for Total Organic Content

Extracts and organic liquid samples are analyzed for total organic content using analytical methods recommended in the IERL-RTP Procedures Manual (1). Samples can contain both moderately volatile organic compounds (bp 100° to 300°C) and nonvolatile organics (bp >300°C). Organic content of extracts must be determined by the appropriate methods (below) before, and possibly after, any procedure which requires solvent evaporation.

Total Chromatographable Organics (TCO) Analysis. Quantitative anal-ysis of moderately volatile materials is achieved by a gas chromatographic procedure called total chromatographable organics (TCO) analysis (Section 9.4.1, Reference 1). TCO is used only with SASS and aqueous-sample sorbent-resin extracts. TCO analysis is not appropriate for samples that have been collected or stored under conditions which permit volatilization of low to moderate boiling organics.

Because materials in the TCO volatility range may be lost to varying degrees during solvent evaporation, it is important that this analysis be performed on extracts and solutions before and after any concentration step.

Gravimetric (GRAV) Analysis. Nonvolatile organic components in all extracts are quantitated by gravimetric (GRAV) analysis (Section 9.4.2., Reference 1). This simple procedure consists of taking an aliquot of the sample and evaporating it in a preweighed aluminium weighing pan. The sample is dried to constant weight (±0.1 mg) and the residue weight determined. The GRAV nonvolatile organic content is calculated from the residue weight and reported as one number for the whole sample. At least 10 mg of sample residue should be weighed, but no more than 10 percent of the sample should be used for GRAV analysis. This procedure is suitable for all extract sample types including extracts from SASS particulates and residues from high-temperature processes. The GRAV analysis method is also applicable for solid-weight determination of slurries. Highly viscous liquids and pastes are weighed directly, placed in a suitable solvent and dosed on a weight-per-volume basis.

2.3.6 Concentration Of Extracts and Solvent Exchange Procedure*

It will usually be necessary to concentrate the organic material from sorbent-resin extracts to a volume of 10 ml for subsequent analysis. Total organic content of the extract is determined by GRAV and TCO analysis (Section 2.3.5) before and after solvent evaporation. It is recommended that concentration to slightly less than 10 ml volume (i.e., 8 or 9 ml) be accomplished using a Kuderna-Danish (K-D) apparatus with a three-ball

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*For all extracts except SASS particulate fractions and residues from high temperature process.

Snyder column for volumes less than 1 liter. For volumes greater than 1 liter, a rotary evaporator should be used to reduce the initial volume to approximately 100 ml. The resulting sample may be concentrated further by K-D. It is essential that the extract not be reduced to dryness at this point in the scheme to prevent loss of volatile material. The concentrated extract should then be transferred to a graduated container (e.g., Kuderna-Danish receiver or centrifuge tube) and the volume restored to 10 ml.

The concentration process should be stopped if material begins to drop out of solution. In that case, the extract should be restored to a convenient volume in which the material is redissolved.

Bioassay testing requires that the dichloromethane solvent be eliminated before the sample extract is applied to the test system. The appropriate volume of extract is carefully reduced to 1 ml at ≤40°C under a gentle stream of nitrogen (tapped from a liquid-nitrogen cylinder, if possible, to minimize impurities). The solvent evaporates rapidly, so it is important that this operation be done under constant surveillance to ensure that the volume is not reduced below 1 ml. It is also necessary to warm the samples slightly, either by hand or water bath at ≤40°C, to prevent condensation of atmospheric moisture in the sample caused by evaporative cooling.

One milliliter of DMSO is added and mixed by gentle agitation. The volume is reduced to a total of 1.5 ml. Another 1 ml of DMSO is added, mixed and the volume is reduced to 2.25 ml. The exchange is repeated with another 1 ml of DMSO and the volume is reduced to a final volume of 3 ml. Other DMSO volumes may be used if 3 ml of DMSO does not give a suitable sample preparation.

2.3.7 Particulate Removal From Glass Mat Filters

The 1 μm or less fraction of SASS train particulate samples is often supplied for biological testing still embedded on the surface of a glass-mat filter. The purpose of this procedure is to optimally remove particulate on glass mat filters while minimizing glass-shard removal.

Filters should be cleaned before sampling to remove shards from the filters. This is done by sonicating blank filters in an ultrasonic water bath* for two hours in cyclohexane. Filters should be placed in sterile glass dishes, large enough so the filter is not bent, and covered by at least 3 cm of solvent. Standard 142-mm filters (such as Reeve Angel 934 AH filters) can be cleaned individually in 150-mm x 75-mm crystallizing dishes using approximately 400 ml of cyclohexane. After cleaning, the filters are removed to a clean, lint-free surface to dry. Once dry, the filters are dessicated for at least 12 hours, filters are weighed then stored in labeled petri dishes. Care should be taken in handling cleaned and loaded filters to mimimize the release of glass shards to particulate samples.

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*For example, Sonicor, Sonicor Instrument Corporation, Copiague, NY 11726.

Each filter is placed in a sterile glass dish (such as that used in conditioning) and cyclohexane added to a depth of approximately 1 cm (200 ml in the 150-mm x 75-mm crystallizing dish). The dish is placed in the ultrasonic cleaner (with at least 4 cm of bathwater or as recommended by the manufacturer) and sonicated for five minutes. The filter is removed to a clean, lint-free surface loaded side-up, and covered with a clean paper towel.

Solvent is transferred to a 500-ml, round-bottom flask using a 25-ml solvent rinse of the dish. The filter is sonicated in cyclohexane for a second five minute period. The solvent and rinse from the second sonication is combined with the solvent obtained from the first wash. Multiple filters are occasionally supplied as one test sample. Particulate suspensions from multiple-filter samples are combined in one common round-bottom flask. The suspension is evaporated with a rotary evaporation apparatus between additions so that no more than 300 ml are in the flask at any one time.

The particulate suspension, whether from one filter or combined from several, is evaporated to a small volume with a rotary evaporator. The concentrated particulate suspension is then transferred to a tared, amber-glass vial. In transferring the concentrated suspension to the vial, several sonications using fresh cyclohexane may be necessary to clean the round-bottom flasks. The solvent is evaporated to dryness under a stream of nitrogen in a warm-water bath. The dried residue in the vial is then dessicated for at least 12 hours and then weighed. The weight gain of the vial is the weight of particulate material available for testing.

The residue is resuspended in a volume of DMSO to give the desired concentration of particulate. Vial caps should be lined with sterile Teflon rounds. Samples suspended in DMSO are stored, tightly capped at +4°C until used.

2.3.8 Concentration of Aqueous Samples By Lyophilization

Aqueous samples supplied for biological testing are often collected from receiving bodies of water or other sources of water anticipated to have low toxicant levels. Testing aqueous samples for toxicity in the acute *in vivo* rodent toxicity assay often requires samples to be concentrated to bring toxicants above the threshold of assay sensitivity. Corrections are made in evaluating sample toxicity to compensate for the effects of concentration.

Aqueous samples for rodent toxicity testing are concentrated 4- to 10-fold by lyophilization. An aliquot of the sample (commonly 500 ml) is taken and frozen in a chemically clean, bulk-freeze-drying flask. The sample is reduced to a little less than one tenth the initial volume using a large-sized lyophilyzing apparatus* at -40°C with a vacuum of 100 microns

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*For example, Virtus Company, Inc., Gardiner, NY 12525.

Hg. The sample is then thawed and transferred with deionized or glass-distilled-water rinses to a clean, graduated cylinder. The volume is increased to the desired volume (usually 50 ml) with deionized or glass-distilled water. The sample is transferred to an amber-glass, screw-top bottle and stored at +4°C. The container label should provide full information about the original sample as well as the concentration process.

2.3.9 Leachate Preparation

This procedure is used to leach water-soluble components from solid samples. Aqueous leachates are prepared by shaking a known weight of solid with distilled water and separation of the aqueous phase by filtration.

Solid test samples are ground, if necessary, so as to pass through a 9.5 mm (3/8 in.) standard sieve. Drying of the sample is not recommended as volatile components may be driven off. A representative portion of the test material is weighed and placed into the container to be used for leaching. A volume of dilution water is added to the container equal (in milliliters) to four times the weight (in grams) of sample. Large volumes of leachate maybe prepared in a 30-gallon, linear polyethylene drum. The drum is placed on a drum roller and agitated for 48 hours at 20 ± 2°C. Smaller containers may be used if less leachate is required. Agitation equipment should produce constant movement of the aqueous phase equivalent to that of a reciprocating platform shaker operated at 60 to 70 (25-mm: 1-in.) strokes per minute. The suspension is allowed to settle after 48 hours of agitation. The aqueous phase is separated from any solid or nonaqueous phases by decantation, centrifugation or filtration through filter paper, as appropriate. The aqueous phase is vacuum- or pressure-filtered through a 0.45-μm-membrane filter and stored in a sterile container of a size such that the entire bottle is filled.

2.4 SPECIAL PROBLEMS

Certain types of samples present testing problems. Slurry samples present unique problems since they tend to precipitate in DMSO. This is particularly troublesome at high concentrations in the *in vitro* clonal cell assay. A layer of precipitate covering the cells may result in death caused by physical problems; solids may resist normal washing procedures or may interefere with scoring by obliterating the colony.

Many particulate samples derived from the combustion process contain adhered toxic chemicals which are only slowly extracted from the particles by most bioassay systems. Preliminary extraction using organic solvents (e.g., dichloromethane) can be used to acquire concentrated volumes of the adhering chemicals. However, this approach may have little relevance to normal *in vivo* toxicity; it may skew the bioassay responses and identify most combustion process streams as highly toxic and candidates for higher level testing. Bioassay of extract from particulate samples is not generally required for Level 1 assessment but may be done *in addition* to testing the whole particulate for comparative purposes (see Table 2.4).

2.5 SAMPLE AMOUNTS REQUIRED FOR LEVEL 1 BIOASSAYS

A frequent problem encountered in environmental assessment is the availability of sufficient sample to conduct all of the desired tests. Table 2.6 lists each test conducted in Level 1 and the anticipated amount of sample needed. It must be kept in mind, however, that the level of toxicity will determine the final amount required. The amount of test material, in some cases, is also dependent upon the characteristics of the sample. For example, the maximum applicable dose (MAD) and the volume of nonaqueous liquid samples required for aquatic ecological testing is dependent upon the solubility determined for each sample before testing is initiated.

The use of weights or volumes in test systems must be uniform. To achieve this uniformity, the following rules will be used to direct the units of concentration:

1. A sample with a density approximating that of water and no suspended particles will be tested as a liquid, using microliters (μl) as the unit of volume.

2. A sample which is a solid or a slurry consisting of $\geq$ 5 percent of the total as solids will be tested as a solid material using micrograms (μg) as the unit of weight.

3. A suspension which contains less than 5 percent of its total as a solid will be tested as a liquid.

4. SASS sorbent resin extracts in DMSO will be tested as a nonaqueous liquid using microliters as the unit of volume. However, the dose will also be calculated based on the concentration of organics using μg as the unit of weight. An attempt will also be made to calculate the volume of original gas sampled per unit weight of organics.

TABLE 2.6 ANTICIPATED SAMPLE AMOUNTS REQUIRED TO CONDUCT LEVEL 1 TESTS

Bioassay	Solid (grams)	Liquid (milliliters)		Gas (liters)
		Aqueous	Nonaqueous[a]	
HEALTH EFFECTS				
Ames Salmonella	2.0 (.5)[b]	5 (1.5)[c]	5 (1.5)	d
RAM Toxicity	0.1 (.025)	45 (15)	2 (0.6)	---
CHO Clonal Toxicity	0.1 (.025)	45 (15)	2 (0.6)	---
Rodent Toxicity	10.0 (5)	50 (25)[e]	20 (10.0)	---
AQUATIC ECOLOGICAL EFFECTS				
Freshwater Fish	10 kg (7.5 kg)	40L (30L)	1L (750)[f]	---
Freshwater Invertebrate	0.5 kg (0.3 kg)	2L (1.5L)	200 (150)	---
Freshwater Algae	0.25 kg (0.13) kg	1L (0.6L)	100 (60)	---
Marine Fish	10 kg (7.5 kg)	40L (30L)	1L (750)	---
Marine Invertebrate	2 kg (1.2 kg)	8L (6L)	800 (600)	---
Marine Algae	0.25 kg (0.13 kg)	1L (.6L)	100 (60)	---
TERRESTRIAL ECOLOGICAL EFFECTS				
Plant Stress Ethylene	d	d	d	1,365
Root Elongation	2.5 kg	10L (5L)	d	---
Insect Toxicity	.5 (.1)	20 (10)	10 (5)	d

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[a] Nonaqueous liquid include samples with greater than 0.2% organics, solvent exchange samples, and extracts.

[b] The first value is the requested sample size for Level 1 testing. The value in parentheses is the minimum feasible sample size to conduct the test.

[c] When concentrated (Section 2.3.4), up to 5 liters (1.5 liters minimum) are required.

[d] Sample form is compatible to bioassay but modifications are beyond the scope of this edition of the Level 1 manual.

[e] When samples are concentrated (Section 2.3.8) up to 0.5 liters (0.25 liters minimum) are required.

[f] The maximum applicable dose (MAD) and the volume of nonaqueous liquid samples required for aquatic ecological testing is dependent upon the solubility determined for each sample before testing is initiated. For additional information, contact the Technical Support Staff, Process Measurements Branch, IERL-RTP, U.S. EPA, Research Triangle Park, NC 27711.

CHAPTER 3

LEVEL 1 HEALTH EFFECTS BIOASSAYS

3.1 INTRODUCTION AND RATIONALE

The Level 1 health effects tests include assays for determining toxicity and mutagenicity at several levels in organisms ranging in complexity from bacteria to mammalian cells in culture (both permanent cell lines and primary cells) to intact animals. Table 3.1 describes the biological characteristics of the target organisms in this group of tests. The tests are able to detect molecular changes such as DNA mutation (Ames test), acute cell toxicity (RAM and CHO tests) and complex toxicological responses in intact animals (WAT test).

Table 3.2 summarizes the types of data obtained, the nature of the observed response and the need for statistical analysis in the Level 1 assays. This group of tests offers broad coverage of toxicity with the concomitant advantages of low cost, reproducibility, rapid performance period and small sample sizes (Table 3.3). These features are consistent with the goals of Level 1 environmental assessment.

Health effects bioassays are used to determine the concentration of test material that produces either a defined mutagenic or toxic effect on the test organisms in a short period of time. The Ames *Salmonella*/ microsome mutagenesis assay (Ames) identifies the minimum effective concentration (MEC) of a test sample that produces significant mutagenesis in any of four tester strains of *Salmonella typhimurium* used.

The rabbit alveolar macrophage assay (RAM) measures four endpoints relating to cell death and metabolic impairment, following 20 hours of continuous exposure. The effective concentration of toxicant that reduces each parameter to 50 percent of the control (EC_{50}) is calculated. The EC_{50} is also estimated in the rodent cell (CHO) clonal toxicity assay based upon the reduction in colony-forming ability of the cells following 24 hours of continuous exposure. Mortality and physiological observations are recorded in both the quantal and quantitative phases of the acute *in vivo* test in rodents (whole animal test, WAT). For samples exhibiting toxicity in the quantal phase, the dose lethal to 50 percent of the animals (LD_{50}) is calculated. Additional health effects endpoints may be measured in modified versions of the CHO toxicity test.

Results from Level 1 health effects tests are interpreted by using evaluation criteria unique to each test. Test samples are ranked according to relative mutagenicity or toxicity using guidelines presented in the results and data interpretation section for each test.

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TABLE 3.1 CHARACTERISTICS OF LEVEL 1 HEALTH EFFECTS BIOASSAYS

Characteristic	Salmonella Mutagenesis	Cytotoxicity Assays		WAT
		RAM	CHO	
Cell Type/Organ System	Prokaryotic Cell-Enteric Bacteria Species	Eukaryotic-Primary Rabbit Macrophage Cells	Eukaryotic-Hamster Cell Line	Integrated Organ and Tissue Systems
End Point(s) Measured	Point Mutation	Lethality and Metabolic Impairment	Cell Lethality	Lethality-Toxic Signs
Amenable to Sample Types	Solids, Liquids, Particulates	Solids, Liquids, Particulates	Solids, Liquids, Particulates	Solids, Liquids, Particulates
Data Expression	Positive or Negative	EC_{50} (Viability, ATP)	EC_{50} (Clonal)	LD_{50} or Toxic Signs
Special Features	Requires *In Vitro* Activation System to Detect Active Metabolites	Especially Effective for Particulate Samples Because Cells Are Phagocytic	Detects effects on Reproductive Capacity of Cells. Same Cells May Be Used For SCE Assay	Can Detect Complex Toxicological Phenomena that Are Dependent on Interactions of Several Organ Systems

TABLE 3.2 LEVEL 1 DATA PRESENTATION

Assay	Type of Data Obtained	Response Summarized	Statistical Analysis
Salmonella Assay	Mutagenic responses in one or more bacterial strains compared against established criteria for a positive effect Determination of the lowest tested concentration giving a response (Minimum Effective Concentration, MEC)	Positive or negative Dose-response effect can be graphed MEC for positive responses Toxicity can be estimated	Not normally performed. However, if each dose is assayed in replicate, a mean ± SD can be calculated
RAM Assay	Trypan blue dye exclusion as an estimate of living and dead cells ATP measurement using a fluorometer Replicate cultures per dose evaluated	EC_{50} values using percent viability and viability index calculated as percent of control (curves are plotted) EC_{50} value using ATP/10^6 cells and ATP/flask calculated as percent of control (curves are plotted)	Mean ± SD calculated for replicate samples EC_{50} and 95% confidence limits can be calculated.
CHO Assay	Number of surviving colonies at each concentration $\text{Survival} = \frac{\text{colonies of treated cells}}{\text{colonies of control cells}} \times 100$ Replicate cloning per dose	Survival curve is graphed EC_{50} value derived from 50% reduction in colonies relative to control	Mean ± SD calculated for replicate samples EC_{50} and 95% confidence limits can be calculated.
WAT Assay	Lethality (LD_{50}) and descriptions of toxic signs	LD_{50} Value derived from 50% reduction in survival	Litchfield/Wilcoxin Analysis for LD_{50}

TABLE 3.3 ADVANTAGES AND LIMITATIONS OF LEVEL 1 HEALTH EFFECTS SCREENING TESTS

Advantages	Limitations
Cost Effective	Extrapolation of Results to Humans Uncertain
Rapid	High Level of Technical Ability Required in Some Assays
Large Data Base for Chemicals Tested	Route of Exposure Not Always Relevant to Human Experience
Good Reproducibility	Prediction of Toxicity *In Vivo* Not Highly Quantitative
Salmonella Assay Correlates with Mammalian Carcinogenesis For Many Classes of Chemicals	Limited Opportunity To Evaluate Volatile Components of Industrial Emissions
RAM Assay Amenable to Study of Particulates	
Assays Represent Sensitive Targets for Toxic Agents	
Use of S9 Mix in *Salmonella* Assay Permits Evaluation of Metabolites	
Small Sample Size Required to Conduct Assay Except for the Rodent Toxicity Assay	
Indirect Mammalian Toxicity (e.g., Neurotoxicity) Can Be Detected in the Rodent Assay	

3.2 AMES SALMONELLA/MICROSOME MUTAGENESIS ASSAY

3.2.1 Introduction and Rationale

The Ames assay is based on the property of selected Salmonella typhimurium mutants to revert from an histidine-requiring state (auxotrophy) to an histidine-synthesizing state (prototrophy) as a result of exposure to mutagens (6). The test is designed to mimic mammalian metabolic processes by the incorporation of a mammalian liver 9,000 X g microsomal fraction (S-9) and the cofactors necessary to generate enzymatic activity. The test is used as a screen for mutagenic activity of pure compounds, complex mixtures or component fractions. It has recently been demonstrated that most initiating carcinogens have mutagenic activity. In several comparative studies the Ames assay has demonstrated approximately 80 to 85 percent accuracy in detecting known animal carcinogens as mutagens (7). However, some known carcinogens are negative in the test (e.g., diethylstilbestrol, natulan, asbestos and some carcinogenic metals) or only very weakly positive. This may be because many of the negative agents are not believed to be initiating carcinogens (8). Chemicals which are mutagenic in the Ames system but have not been shown to be carcinogenic in mammals are also known. These mutagens may represent a unique class of chemicals, or the animal tests may not be sufficiently sensitive to detect their carcinogenic effect. Continued improvement of the present bacterial strains, addition of new strains, standardization of the Salmonella assay and re-evaluation of the conventional animal carcinogenesis data may reduce this level of error; however, a perfect correlation between mutation and carcinogenesis is unlikely. The following discussion is intended as a general description of the test; a detailed study design is presented in the published method (6).

3.2.2 Materials and Methods

Indicator organisms. The indicator organisms to be used are the Salmonella typhimurium tester strains developed by Dr. Bruce Ames (TA-1535, TA-1537, TA-98, and TA-100). They are histidine-deficient variants and are used to detect reverse mutations, which are either frameshift (TA-1537 and TA-98) or base-pair substitutions (TA-1535 and TA-100), as indicated by reversion to histidine prototrophy (Table 3.4).

Liver microsome preparations. The activation system for mutagenesis screening consists of Aroclor 1254, induced S-9 fraction derived from rat livers. Male, Sprague-Dawley rats weighing approximately 200 g each are used. Induction is accomplished by a single intraperitoneal injection of Aroclor 1254 (diluted in corn oil to 200 mg/ml) into each rat five days before sacrifice at a dosage of 0.5 mg/g of body weight. All rats are deprived of food (not water) 12 hours before sacrifice.

The following steps are carried out at 4°C using cold sterile solutions and glassware. The livers (10 to 15 g) are aseptically removed from the rats and placed into a cold, preweighed beaker containing 10 to 15 ml of 0.15 M KCl.

TABLE 3.4 SALMONELLA TYPHIMURIUM STRAIN CHARACTERISTICS

Strain Designation	Gene Affected	Additional Mutations[a]			Mutation Type Detected
		Repair	LPS	R Factor	
TA-1535	his G	Δ uvr B	rfa	-	Base-pair substitution
TA-1537	his C	Δ uvr B	rfa	-	Frameshift
TA-98	his D	Δ uvr B	rfa	pKM101	Frameshift
TA-100	his G	Δ uvr B	rfa	pKM101	Base-pair substitution

[a] Reference 6 presents a discussion of the additional mutations and description of the abbreviations.

After the livers are washed and weighed in this beaker, they are removed with forceps to a second beaker containing 3 ml of the KCl solution per gram of wet-liver weight. The livers are then minced with sterile scissors, transferred to a chilled glass homogenizing tube, and homogenized in an ice bath by passing a low-speed motor-driven pestle through the livers a maximum of three times. The chilled homogenates are then placed into centrifuge tubes and centrifuged for 10 minutes at 9,000 X g at 4°C. The resulting supernatant is decanted, transferred in 2-ml amounts to small storage tubes, quickly frozen in dry ice and stored at -75° to -80°C in a low-temperature freezer. This supernatant is known as the S-9 fraction. Sufficient S-9 for use each day is thawed at room temperature and kept on ice before and during use. The extent of bacterial contamination of the S-9 fraction should be determined. The S-9 mix may be filter sterilized (0.45 μm porosity filter) if required.

The quality of each S-9 lot is determined before the lot is released for general use. The enzymatic activity of S-9 is measured by testing reference mutagens, such as benzo-a-pyrene or 2-anthramine, in the Ames assay. Metabolic activation is required for these chemicals to be detected as mutagens. The protein content of the S-9 is also determined. The normal range of values is 28 to 45 mg of protein per milliliter of S-9.

Metabolic activation mixture. The S-9 microsomal mix is prepared according to the recommendations of Ames described earlier. The mix contains per ml: S-9 (0.1 ml), $MgCl_2$ (8 μmoles), KCl (33 μmoles), D-glucose-6-phosphate (5 μmoles), nicotinamide adenine dinucleotide phosphate (NADP) (4 μmoles) and sodium phosphate, pH 7.4 (100 μmoles). The S-9 mix is prepared fresh each day and is maintained on ice before and during use. Use of the S-9 mix should not exceed 6 hours at 0°C.

Bacteriological media. The minimal-glucose agar medium for histidine-requiring strains used in mutagenesis assays is a 1.5 percent Difco-Bacto agar in Vogel-Bonner Medium E with 2 percent glucose. Top agar (0.6 percent purified agar, 0.1 M NaCl) contains 0.05 mM histidine and 0.05 mM biotin to permit the bacteria to undergo several divisions.

3.2.3 Experimental Design

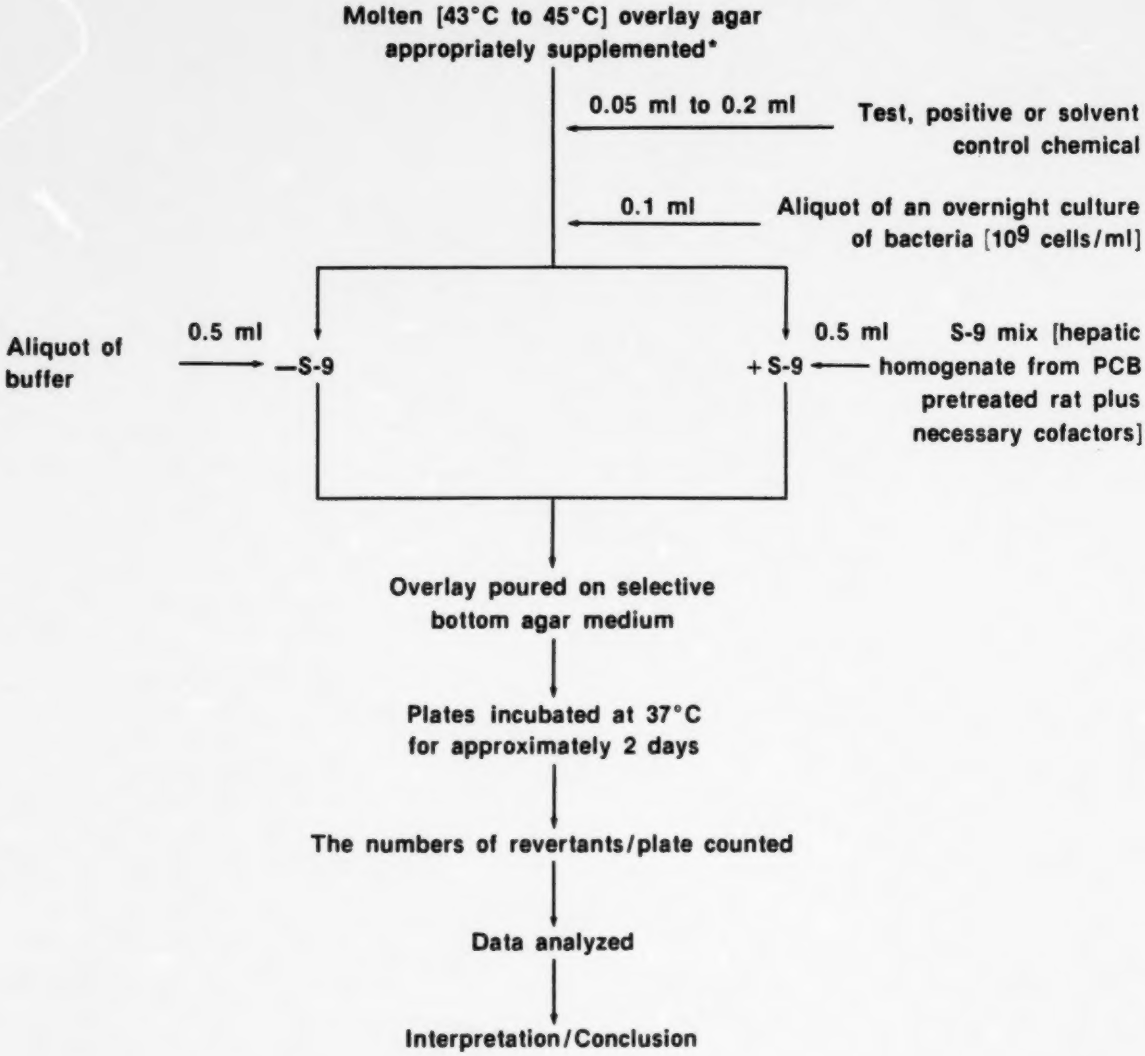
General test procedures. The plate-incorporation version of the Ames assay is recommended for routine use in Level 1 mutagenicity assessment. In the plate-incorporation assay, the sample is added directly to molten top agar (45°C) which is then poured onto the plates along with the test organism and the liver S-9 activation system (Figure 3.1). Test samples are diluted in water, dimethylsulfoxide, ethanol or acetone so that constant volume aliquots are added to each plate. The solvents are listed in order of preference. Maximum liquid volume of test sample and vehicle should not exceed 0.2 ml per plate. Once the overlay has solidified, the plates are incubated at 37°C for 48 to 72 hours. Typically, 48 hours is specified unless there is evidence of growth inhibition. Plates are then scored for the number of revertants per plate.

It is recommended that the standard plate-incorporation assay be performed in duplicate for each test concentration and control. Controls for each test include solvent or vehicle controls to measure the spontaneous reversion frequency for each tester strain. A sterility check of the test-material solution or suspension is made. Positive controls consisting of compounds which both do and do not require metabolic activation are conducted concurrently with each assay. Quality control procedures are summarized in Chapter 7 and detailed in Reference 9.

Testing strategy and dose selection are dependent upon sample type and availability. If test material is limited, it is advisable to test test agents with the Salmonella strains TA-98 and TA-100 only prior to conducting complete mutagenic assays. This step may reduce the amount of chemical used if the test material proves to be highly toxic or mutagenic. Solid samples are tested for mutagenicity at the maximum applicable dose (MAD) of 5 mg per plate and at five lower concentrations of 2.5, 1, 0.5, 0.1 and 0.05 mg per plate. Additional sample types that are also tested on a weight-per-volume basis are slurries and sample extracts of known organic content. If the initial test is negative, it should be repeated after one week for confirmation with strains TA-98 and TA-100. If the results are positive, repeat studies should be performed over a narrower concentration range with strains showing positive results in the initial test to identify the minimum effective concentration (MEC). Solid samples may be tested up to 10 mg per plate if there is evidence of a weak-positive effect at 5 mg/plate. The requirement for retesting is dependent upon the availability of sufficient quantities of test samples.

All nonaqueous liquids are tested in a minimum assay starting at the MAD concentration of 200 µl per plate, and at lower concentrations of 100, 50, 10, 5 and 1 µl/plate. Samples are retested after one week if the

AMES ASSAY [PLATE INCORPORATION METHOD]


Molten [43°C to 45°C] overlay agar appropriately supplemented*
0.05 ml to 0.2 ml
Test, positive or solvent control chemical
0.1 ml
Aliquot of an overnight culture of bacteria [10^9 cells/ml]
Aliquot of buffer
0.5 ml
—S-9
+S-9
0.5 ml
S-9 mix [hepatic homogenate from PCB pretreated rat plus necessary cofactors]
Overlay poured on selective bottom agar medium
Plates incubated at 37°C for approximately 2 days
The numbers of revertants/plate counted
Data analyzed
Interpretation/Conclusion

[*] A modification of this test called the "Preincubation Modification" consists of a 15-20 minute preincubation of the cells, chemical and S-9 at 37°C before the overlay is added. Certain agents not active in the standard method will be positive in this modification.

Figure 3.1 AMES SALMONELLA/MICROSOME MUTAGENESIS ASSAY

test is negative with strains TA-98 and TA-100. Mutagenic samples are retested over a narrower range of concentrations with those strains showing positive results initially to identify the MEC.

A modification to the Ames mutagenesis assay is used to test receiving-body-water samples and other dilute aqueous samples. Frequently mutagens occur in environmental samples below the threshold of sensitivity for many classes of chemicals in the Ames test. Aqueous samples often need to be concentrated to detect mutagenic activity. Testing concentrated aqueous samples may appear to contradict the screening nature of Level 1 assessment. However, pretest processing is acceptable to enhance the sensitivity of the Ames test because of the need for ranking of process streams, for confirming investigations and/or for control technology application.

Testing strategy requires aqueous samples to be tested at the MAD of 200 µl per plate. If the neat sample at 200 µl per plate is mutagenic, a repeat test is performed over a narrower dose range with strains showing a mutagenic response to identify the MEC. Aqueous samples that are negative in the minimum assay are concentrated 1000-fold on sorbent resins as discussed in Sections 2.3.4, 2.3.5, and 2.3.6. The sample extract in DMSO is tested over the range of 200, 100, 50, 10, 5, and 1 µl per plate. Samples that are negative are retested one week later with strains TA-98 and TA-100; those that are positive are retested with a narrower range of concentrations with strains giving positive results if the MEC is not identified in the initial assay.

Positive controls. Positive-control mutagens are run with each strain. Both direct-acting compounds and compounds requiring activation are used. Each positive-control chemical has a preferred solvent. Untreated controls using the appropriate solvent are run for each indicator strain. Table 3.5 lists the chemicals, solvents and concentrations which may be routinely used as positive controls for each strain.

TABLE 3.5 POSITIVE CONTROL MUTAGENS

Assay	Chemical	Solvent	Concentration per Plate (ug)	Salmonella Strains
Nonactivation	Sodium azide	Water	10	TA-1535, TA-100
	2-nitrofluorene (NF)	Dimethyl-sulfoxide	10	TA-98
	9-aminoacridine (9AA)	Ethanol	50	TA-1537
Activation	2-anthramine (ANTH)	Dimethyl-sulfoxide	2.5	TA-1535, TA-1537 TA-98, TA-100

Negative controls. Both a negative (untreated cells) and a solvent (test sample vehicle) control are conducted concurrently with each assay. The concentration of solvent used in the solvent control is equal to the maximum concentration of solvent used in dosing the test material. If no solvent is used, only the untreated control is conducted.

Modifications of the assay. In addition to the plate-incorporation method of analysis, other procedures may be of value in chemical assessment. Certain types of materials such as dialkylnitrosamines and hydrazines are not active in the standard method but require a preincubation of the test agent, S-9 mix and indicator organisms for approximately 30 minutes at 37°C prior to the addition of overlay agar (10). Selection of the preincubation modification should be made after discussions with the source contact regarding the chemistry of the sample or under direction from the EPA Project Officer.

3.2.4 Results and Data Interpretation

Acceptance of test data is based primarily on control test results. Negative or solvent controls for each strain should have a background mutant frequency similar to the ranges presented in Table 3.6. Positive controls should give a positive mutagenic response when analyzed by the evaluation criteria that follow.

TABLE 3.6 ACCEPTABLE SPONTANEOUS REVERTANTS PER PLATE

Strain	Revertants/Plate
TA-1535	20 ± 10
TA-1537	15 ± 10
TA-98	50 ± 25
TA-100	150 ± 75

Because the procedures to be used to evaluate the mutagenicity of the test article are semiquantitative, the criteria to be used to determine positive effects are inherently subjective and are based primarily on a historical data base.

Plate-test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test article, the surviving population on the treatment plates is essentially the same as that on the negative-control plate. At high concentrations, the surviving population is usually reduced by some fraction. One requirement in evaluating Ames mutagenesis data is that the selected doses range over at least two log concentrations, the highest of these doses being selected possibly to show slight toxicity as determined by subjective criteria.

Mutagenicity is evidenced by reversion to prototrophy and colony formation on the selective culture medium. A sample may be considered mutagenic if the number of induced revertants is more than three times the solvent-control value for strains TA-1535 and TA-1537 or more than two times the solvent-control value for strains TA-98 and TA-100.

The demonstration of dose-related increases in mutant counts is an important criterion in establishing mutagenicity. Generally, a positive dose-response over three test concentrations should be observed in conjunction with the relative fold increases identified above. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test article may kill any mutants that are induced, and the test article will not appear to be mutagenic.

The goal of Level 1 Ames testing is to rank source streams by relative degree of genetic toxicity (mutagenicity). Samples identified as mutagenic by the criteria above are then ranked by the evaluation criteria presented in Table 3.7. The lowest concentration giving a positive response in any strain is identified as the minimum effective concentration (MEC) for that sample. The final report should identify the MEC and mutagenicity category for each sample. Samples with no detectable activity at the maximum applicable dose (MAD) are ranked not detectable (ND). A convenient method to derive the MEC is to graphically represent the dose-response data corrected for the spontaneous background. The MEC is the concentration required to induce a two- (TA-98 and TA-100 strains) or three-fold (TA-1535 and TA-1537) increase over the spontaneous value. The MEC value should fall on the linear part of the dose response curve.

TABLE 3.7 AMES ASSAY EVALUATION CRITERIA

Mutagenic Activity	Solids (MEC in mg/plate)	Liquids (MEC in µl/plate)	Organic Extracts[a] (MEC in µl/plate)
High	<0.05	<2	<2
Moderate	0.05-0.5	2-20	2-20
Low	0.5-5	20-200	20-200
Not dectable	>5	>200	>200

[a] Consult Section 3.2.4 for explanation of evaluation criteria for aqueous sample extracts.

Aqueous samples are evaluated in a slightly different manner from solids and nonaqueous liquids. Aqueous samples are evaluated initially as unconcentrated liquids. Those samples ranked as nondetectable are then concentrated 1000-fold and tested as an organic extract. The processed sample is then ranked by the criteria in Table 3.7 under organic extract. Reports will include the data and final evaluation for both the unconcentrated and the organic extract. The ranking of organic extracts of aqueous samples is meaningful only to similar sample types; comparison of data to other sample types is not recommended.

3.3 RABBIT ALVEOLAR MACROPHAGE (RAM) CYTOTOXICITY ASSAY (11-15)

3.3.1 Introduction and Rationale

Primary cell cultures of rabbit alveolar macrophages (RAM) are used to measure cell death and metabolic impairment resulting from *in vitro* exposure to particulate and soluble toxicants. The RAM cells constitute a first line of pulmonary defense because of their ability to engulf and remove particulate materials which are deposited in the lung. Primary RAM cell cultures exhibit many of the metabolic and functional attributes of the original tissues. Therefore, it is appropriate that this cell type be used to define the acute cellular toxicity of airborn particulates and associated chemicals as part of the Level 1 health effects environmental assessment.

The RAM cytotoxicity assay has been employed effectively in cytotoxicity screening of a wide variety of pure chemicals, mixtures and environmental contaminants. Recently, this system has been applied in evaluating the relative cellular toxicities of hazardous metallic salts (11) and particulate effluents from coal gasification, fluidized bed combustion and conventional coal-combustion processes (12). This cytotoxicity screening system has also been used to "rank" the toxicities of industrial particulates collected from SASS cyclone sampling trains. Compared to conventional, whole-animal tests for acute toxicity, this assay is more rapid, less costly and requires significantly less sample. However, the assay employs isolated cells rather than intact animals, so the information may be difficult to use to predict health hazards of toxic chemicals.

The standard RAM assay is used to develop cytotoxicity data following 20 hours of continuous exposure to test materials. The observed endpoints are cell viability as measured by trypan blue dye exclusion and metabolic impairment as measured by cellular adenosine triphosphate (ATP) levels. Results are expressed in terms of an EC_{50} concentration, which is the estimated test concentration causing a 50 percent reduction in each measured parameter relative to the control. Statistical methods are recommended for the calculation of the EC_{50} values and the 95 percent confidence limits for each EC_{50} determination.

The RAM assay is used primarily for solid or particulate samples for Level 1 assessment. Aqueous and nonaqueous liquids are compatable with this test but are normally tested in the rodent cell (CHO) clonal toxicity assay discussed in Section 3.4.

3.3.2 Materials and Methods

Indicator cells. This assay employs short-term primary cultures of alveolar macrophages obtained by lung lavage of male and female New Zealand white rabbits. The rabbits are individually housed and fed antibiotic-free laboratory rabbit chow and water *ad libitum*. The animals are examined for the absence of respiratory illness and for over-all general health prior to collection of macrophage.

Media. The cells are maintained and treated in Eagle's Minimum Essential Medium (EMEM) with Earle's salts supplemented with 10 percent fetal bovine serum (heat-inactivated), 100 units/ml penicillin, 100 μg/ml streptomycin and 0.5 μg/ml amphotericin B (Fungizone). Kanamycin (35 μg/ml) may also be added as extra protection against bacterial contamination. Intermediate steps require EMEM with 0 and 20 percent fetal bovine serum (FBS).

An alternate medium, Medium 199, may be substituted for EMEM and supplemented in the same manner. This medium is also available commercially as a 10x concentrate.

Test material. Solid samples are tested as supplied or are finely ground to 5 μm or less (see Section 2.3.1) and tested as a suspension in culture medium. Dry particulate material, aqueous liquids, suspensions and slurries are added directly to the culture medium and tested as a suspension or solution. Liquids containing less than 0.2 percent organic solvent can generally be tested directly. Samples dissolved in organic solvents are solvent-exchanged into dimethylsulfoxide (DMSO) according to Section 2.3.6 before testing.

3.3.3 Experimental Design

Dosage selection. Test materials are pre-screened in triplicate at the maximum applicable dose (MAD) of 1000 μg/ml for solids and particulates, 600 μl/ml for aqueous samples or 20 μl/ml for nonaqueous liquids and sorbent extracts in DMSO. Testing will be terminated and the sample categorized as having toxicity that is not detectable (ND) if no parameter is depressed 50 percent or more relative to the control. If any parameter is depressed 50 percent or more, a definitive test is undertaken with another population of macrophage. Solid and particulate samples are tested in triplicate using a dose range which includes 1000, 500, 100, 50 and 10 μg/ml. Aqueous samples are tested unfiltered (or filtered-through a 0.45 μm membrane filter if necessary) at concentrations of 600, 200, 60, 20 and 6 μl/ml. Nonaqueous samples solvent exchanged into DMSO are tested in triplicate at 20, 10, 5, 1 and 0.5 μl/ml. Dosage and evaluation of organic extracts are also based upon μg/ml of organics when organic content of a sample is known.

Routine pre-screen testing at MAD concentrations is efficient for the testing of multiple samples. The pre-screen test may be eliminated in situations where the sample size is limiting or where only a single sample is to be tested. The dose range may be modified to accomodate sample characteristics or previously gathered toxicity data. Inclusion of additional dose-levels or replicas per dose may be desirable.

Controls. In all cases, triplicate cultures of untreated cells are established and analyzed as the control. If the test material is dissolved in an organic solvent (normally DMSO), cultures exposed to the solvent will constitute the control values. The final concentration of solvent in the growth medium shall be one percent or less, with the exception of the highest dose which shall have no more than two percent solvent.

Procurement of cells. Macrophages are collected from healthy rabbits weighing 1.5 to 2.5 kg. Animals are sacrificed by injection of sodium pentoborbital (60 mg/kg) into the marginal ear vein. The neck and chest area are irrigated with 70 percent ethanol and a tracheostomy is performed using sterile operating procedures. Thirty ml of 0.85 percent saline (23°C) are instilled into the lungs via a catheter and allowed to remain for 15 minutes. The lavage fluid is removed with a sterile 50-ml syringe and placed into a sterile 50-ml centrifuge tube on ice. Five to nine subsequent lavages are performed and fluid is collected, except the saline is removed shortly after its introduction into the lungs. Any tubes of lavage fluid found to contain blood or mucous are discarded.

The cells are centrifuged at 450 x g for 15 minutes, preferably at 4°C. The supernatant is aspirated, the cells resuspended in fresh cold saline, and the cells centrifuged again. After the second centrifugation the cells are resuspended in EMEM containing 20 percent heat-inactivated fetal bovine serum (FBS) and are pooled. Differential cell counts are performed using Wright stain; a minimum of 200 cells is counted. Cells are also counted by hemocytometer and viability determined using trypan blue dye exclusion.

Macrophage populations are discarded if specific parameters are not in the normal ranges. The macrophage fraction of the pooled lavage fluid should be greater than 90 percent of the nucleated cells. Cells are discarded if viability, as determined by trypan blue dye exclusion, is substantially less than 95 percent. The normal yield per rabbit is approximately 30 x 10^6 cells; occasionally up to 50 or 60 x 10^6 cells are obtained (12). If animals yield more than 60 x 10^6 or less than 20 x 10^6 cells, the collection is discarded.

Treatment of cells. Cells are diluted with EMEM (20 percent FBS) to between 5 x 10^5 and 1 x 10^6 cells per ml. Aliquots of cell suspension (2 ml) are then added to each pregassed (5 percent CO_2) test flask. Three flasks are prepared for each concentration and control.

Test samples, whether for the initial prescreen at the MAD concentration or the definitive test, are processed by appropriate pretest procedures discussed in Section 2.3. In addition, particulate samples are leached before testing. Particulate samples are weighed, vortexed dry for two minutes to break up aggregates and are suspended in EMEM (0 percent FBS) at 2000 µg/ml. The particulate suspension is preincubated for 20 hours on a rocker platform at 37°C in a 5 percent CO_2-humidified atmosphere. For all test samples, dilutions are performed with EMEM (0 percent FBS) to twice the desired final concentration for each dose level. Two ml of sample are added per culture flask. Each flask therefore contains between 1 and 2 x 10^6 cells in 4 ml of EMEM containing 10 percent serum and the test agent at the desired final concentration.

A different procedure is used for preparing the high dose (600 µl/ml) of aqueous test samples. To achieve this high concentration, 10x EMEM (or 10x Medium 199, if this alternate medium is used for the entire assay) is diluted with the test sample, the cell suspension and the medium supplements.

If the test substance causes a color change in the culture medium, the pH is determined in additional treated flasks. After the exposure period, the pH of the medium in the experimental flasks is again recorded. If the sample causes a drop in the pH below 6.8 or an increase above 7.6, the sample is retested under pH-adjusted conditions. The pH is adjusted to pH 7.2 using ultra-pure HCl or NaOH.

The dosed flasks are incubated at 37°C with loosened caps in a 5-percent CO_2-humidified atmosphere. After setting for 30 minutes to permit attachment, the flasks are rocked on a platform rocker (12 oscillations per minute) for the remainder of a 20-hour exposure period.

Cell viability assay. At the end of the treatment period, the medium containing unattached cells is decanted into a centrifuge tube on ice. The attached cells are rinsed with 1 ml of 0.1 percent trypsin with 0.01 percent EDTA and then incubated with 2 ml of the trypsin/EDTA solution for approximately 5 minutes at 37°C. The trypsinate rinse and decanted medium are combined for each culture to yield a 7-ml cell suspension for subsequent analyses.

A 1.0-ml aliquot is removed for cell count and viability determination. The aliquot is combined with 0.5 ml of 0.4 percent trypan blue and counted by hemocytometer after five minutes. A minimum of 200 cells is counted. The percent viability is determined for each dose level and compared to the control. The number of viable cells at each dose level is also compared to the number of viable cells in the control to yield the viability index.

ATP assay. ATP is determined using a fluorometer (such as Dupont Model 760 Luminescence Biometer*) according to procedures supplied with the instrument. Aliquots of the cell suspensions are removed and analyzed immediately for ATP content. A 0.1-ml aliquot of cell suspension is added to a 0.9-ml mixture of 90 percent DMSO and 10 percent 0.01 M morpholinopropanesulfonic acid (MOPS) and vortexed for ten minutes. After two minutes at room temperature, 5.0 ml of cold 0.01 M MOPS buffer at pH 7.4 is added and the extract is mixed thoroughly and placed on ice. Aliquots of 10 µl are injected into cuvettes containing 0.7 mM crystalline luciferin, 100 units luciferase, 0.01 M magnesium sulfate, and 0.01 M MOPS buffer (pH 7.4) at 25°C in a total volume of 100 µl. The emitted light is measured photometrically in the luminescence biometer.

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*E.I. du Pont de Nemours and Co., Inc., Wilmington, DE 19898.

The biometer is calibrated daily with standard ATP solutions to provide a direct read-out of the ATP content. Each test sample is assayed at least two times to demonstrate consistent readings.

ATP values are reported as average ATP per flask and ATP per 10^6 total cells for each dose level and both parameters are also expressed as a percent of the control. The dimension used for ATP concentration is femtogram (fg) which is equivalent to 10^{-15} gram.

3.3.4 Results and Data Interpretation

Report. The reports will include the experimental protocol, screened doses, pH values (if appropriate), determination of the EC_{50} values and 95 percent confidence limits for four different parameters and the toxicity ranking of the sample. The EC_{50} value is defined as the test concentration causing a reduction in a measured parameter by 50 percent relative to the control. The four assay parameters are the percent viable cells in the treated cultures, viability index, ATP per culture flask, and ATP per 10^6 cells.

Assay acceptance criteria. The assay will be considered acceptable for evaluation of the test results if the following criteria are met. The macrophage population is 90 percent or greater of the total nucleated cells collected by lung lavage. The percent viability of the macropahges used to initiate the assay is 95 percent or greater. The survival of viable macrophages in the negative control cultures over the 20-hour treatment period is 70 percent or greater. A sufficient number of data points (for five test concentrations or less) is available to clearly locate the EC_{50} of the most sensitive test parameter within a toxicity region as defined under Data Evaluation. The data points critical to the location of the EC_{50} for the most sensitive parameter are the averages of at least two treated cultures. If all the test parameters yield EC_{50} values greater than 1000 µg/ml for solids, 600 µl/ml for aqueous solutions, or 20 µl/ml for organic solutions, the curves for ATP content and viability index parameters do not exceed 120 percent of the appropriate control.

Data evaluation. A substantial quantity of raw toxicity data is produced from the RAM assay. Standard forms should be used to record both raw and analyzed data as recommended in the companion volume giving proposed quality control and quality assurance procedures (9).

Toxicity data can be analyzed using statistical methods developed by Garrett and Stack (16). Four statistical programs are available for a programmable calculator with magnetic-card capability. Data generated by the programs include the mean and standard deviation of replicate values, probability (P) values, the estimated dose level at which a response attains a given magnitude (normally the EC_{50}), and the 95 percent confidence limits for a given EC_{50} determination.

The calculated EC_{50} value for each parameter is evaluated using Table 3.8. The toxicity ranking of the test material is determined by the assay parameter with the lowest EC_{50} value. Organic extracts are evaluated and ranked as solid samples based upon the organic content of the sample (µg organics/ml). The EC_{50} of sorbent extracts of SASS train samples is also converted to the equivalent volume of gas originally sampled.

TABLE 3.8 RAM ASSAY EVALUATION CRITERIA

Toxicity	Solids (EC_{50} in µg/ml)	Aqueous Liquids (EC_{50} in µl/ml)	Nonaqueous Liquids[a] (EC_{50} in µl/ml)
High	<10	<6	<0.2
Moderate	10 to 100	6 to 60	0.2-2
Low	100 to 1000	60 to 600	2-20
Not Detectable	>1000	>600	>20

[a] Criteria for nonaqueous liquids are tentative and under evaluation.

3.4 RODENT CELL CLONAL TOXICITY ASSAY

3.4.1 Introduction and Rationale

Permanent cell cultures of Chinese hamster ovary cells (CHO) are used in this assay for measuring test-material toxicity. The observed end point is the reduction in colony-forming ability of single cells after an *in vitro* exposure to test materials. CHO cells have been widely used in evaluating pure chemicals, mixtures and environmental samples for cytotoxicity.

CHO cells have characteristics which make them suitable for Level 1 toxicity assessment and ranking. The cell line is well characterized and cultures are easily maintained. CHO cells are sensitive indicators of toxicity and provide a good correlation of toxicity with other Level 1 tests. The assay requires only a small amount of sample, is inexpensive and requires only seven days for completion. CHO cells have been shown to be capable of engulfing environmental particulate materials (12). In addition to these characteristics, CHO cells can be easily and inexpensively used to simultaneously measure both genetic toxicity and cytotoxicity (17,18); this capability may be highly desirable when more detailed studies of sample toxicity are required. Suggested procedures to expand the Level 1 CHO toxicity assay to include a genetic end point are discussed in Appendix A.

The CHO clonal toxicity assay is primarily used for aqueous and non-aqueous liquids, but is also compatible with solid and particulate samples. Results are expressed in terms of the EC_{50} concentration, which is the estimated test concentration causing a reduction in colony-forming ability by 50 percent. Statistical methods can be used for the calculation of the EC_{50} value and the 95 percent confidence limits.

3.4.2 Materials and Methods

Indicator Cells. A cell line originally derived from Chinese hamster ovarian tissue and designated CHO-K1 is used for this assay. The cells spontaneously transformed to a stable, hypodiploid line of rounded, fibroblastic cells with unlimited growth potential. Monolayer cultures have a fast doubling time of 11 to 14 hours and normally can be cloned at 80 percent or greater efficiency. Permanent stocks are maintained in liquid nitrogen and laboratory cultures are maintained by serial subculturing. Laboratory cultures are also periodically checked by culturing methods for mycoplasma contamination.

Media. The CHO-K1 cell line has an absolute requirement for proline and therefore must be maintained in a culture medium containing this amino acid. Ham's Nutrient Mixture F12, which contains 3×10^{-4} M L-proline is normally used, supplemented with 10 percent fetal bovine serum and 2 mM L-glutamine. Experimental cultures also contain 100 units penicillin, 100 µg streptomycin and 0.5 µg amphotericin B (Fungizone) per milliliter. A 10x formulation of Ham's F10 medium, which also contains proline, is used for the testing of aqueous samples in order to avoid dilution of medium components.

Test material. Solid samples are either tested as supplied or ground to fine particles (Section 2.3.1). Insoluble solids may be leached by suspending the sample material in Ham's F12 for 20 hours at 37°C on a rocker platform.

Aqueous liquids and suspensions containing less than 0.2 percent organic solvent are directly added by volume to the medium. A 10x concentrate of Ham's F10 is used to prepare the high dose (600 µl/ml) for aqueous liquids and suspensions. Samples supplied in organic solvents, such as sorbent extracts, are usually concentrated and solvent exchanged into DMSO before testing (Section 2.3.6).

3.4.3 Experimental Design

Dosage selection. Dry particulate material is dissolved or suspended in growth medium and tested in triplicate using a dose range which includes 1000, 500, 100, 50 and 10 µg/ml. Aqueous samples are tested unfiltered (or filtered through a 0.45 µm membrane filter if necessary) at concentrations of 600, 200, 60, 20 and 6 µl/ml. Nonaqueous samples and samples that are solvent exchanged into DMSO are tested in triplicate at 20, 10, 5, 1 and 0.5 µl/ml. The dose range may be modified to accommodate sample characteristics or previously gathered toxicity data. Inclusion of additional dose-levels or replicas per dose may be desirable.

Controls. In all cases, untreated cells will be plated to establish the control cloning efficiency. If the test material is dissolved in an organic solvent (usually DMSO), cells exposed to solvent in the medium are cloned to provide the reference cloning efficiency for the effect of the test substance. The final concentration of solvent in the growth medium will be one percent or less with the exception of the highest dose, which will have no more than two percent solvent. All controls are performed in triplicate.

Clonal toxicity assay. Cells from sub-confluent monolayer stock cultures in logarithmic growth phase are trypsinized with 0.25 percent trypsin, counted by hemocytometer, and reseeded into a series of 60-mm culture dishes at 200 cells per dish. The cultures are incubated for 6 to 16 hours at 37°C in a humidified five percent CO_2 atmosphere to allow attachment of the cells and recovery of growth rate.

Test material is then applied (three dishes per dose) and the cultures are returned to the incubator. After a 24-hour exposure period, the medium is aspirated and the cells are washed three times with Dulbecco's phosphate buffered saline (PBS) (prewarmed to 37°C). Fresh medium (5 ml) is placed on each culture and incubation continued for an additional six days to allow colony development. Medium is drained from the cultures and the surviving colonies are washed with PBS, fixed in ethanol and stained with Giemsa. Colony counting is performed manually; colonies smaller than 50 cells are not counted.

If the test substance causes a color change, the pH of the medium is recorded for the doses that produced a color change. At the end of the exposure period, the pH of the discarded medium for which initial pH measurements were made is again recorded. If the sample causes a drop in the pH below 6.8 or an increase above 7.6, the sample is retested under pH-adjusted conditions. The pH is adjusted to pH 7.2 using ultrapure HCl or NaOH.

3.4.4 Results and Data Interpretation

Report. The reports will include the experimental protocol, screened doses, pH values (if appropriate), colony counts, percent survivals (colony counts relative to control colony counts), EC_{50} values with 95 percent confidence limits and toxicity ranking of the sample.

Assay acceptance criteria. The assay is considered acceptable for evaluation of the test results if the following criteria are met. Average cloning efficiency of the CHO-K1 cells in the negative controls is 70 percent or greater, but not exceeding 115 percent. Distribution of colonies in the treated cultures is generally uniform over the surface of the culture dish. Data points for each test concentration critical to the location of the EC_{50} are the averages of at least two treated cultures. A sufficient number of test concentrations are available to clearly locate the EC_{50} with a toxicity region as defined under Data Evaluation. If the EC_{50} value is greater than 1000 µg of solid sample/ml, 600 µl of aqueous sample/ml or 20 µl of nonaqueous sample/ml, the plotted curve does not exceed 110 percent of the negative control.

Data evaluation. Toxicity data can be analyzed using statistical methods developed by Garrett and Stack (16). Four statistical programs are available for a programmable calculator with magnetic-card capability. Data generated by the programs include the mean and standard deviation of replicate values probability (P) values, the estimated dose level at which a response attains a given magnitude (normally the EC_{50}), and the 95 percent confidence limits for a given EC_{50} determination. An alternate method to estimate EC_{50} values is to graphically fit a curve by eye through relative survival data plotted as a function of the logarithm of the applied concentration.

TABLE 3.9 CHO ASSAY EVALUATION CRITERIA

Toxicity	Solids (EC_{50} in μg/ml)	Aqueous Liquids (EC_{50} in μl/ml)	Nonaqueous Liquids[a] (EC_{50} in μl/ml)
High	<10	<6	<0.2
Moderate	10 to 100	6 to 60	0.2-2
Low	100 to 1000	60 to 600	2-20
Not Detectable	>1000	>600	>20

[a] Criteria for nonaqueous liquids are tentative and under evaluation.

The toxicity ranking of the sample is determined by the EC_{50} value and the evaluation criteria in Table 3.9. Organic extracts are evaluated and ranked as solid samples based upon the organic content of the sample (μg organics/ml). The EC_{50} of sorbent extracts of SASS train samples is also converted to the equivalent volume of gas originally sampled.

3.5 ACUTE IN VIVO TOXICITY TEST IN RODENTS

3.5.1 Introduction and Rationale

Because of the complex mixture of chemical compounds in environmental samples and the potential for additive or synergistic action, the rodent in vivo screen is considered to be a valuable test method.

The advantages of the in vivo toxicity assays are embodied mainly in the fact that the toxicological assessment is performed in whole animals. There is a significant background of test data on a wide range of toxicants for the rodent systems, thus supplying needed information for reliable interpretation of results with complex effluents (19).

The main disadvantage of an acute rodent toxicity study is a possibly unsatisfactory prediction of toxicity induced by long-term/low-level exposures. An additional consideration is the need for multi-gram quantities of test material which may prohibit testing where small amounts of sample are available, such as from source streams containing gaseous and particulate material.

Mice and rats are usually the two animals of choice for the measurement of acute toxicity. These choices are made because of the availability of uniform strains, ease of housing, their small size, relatively low cost and a large volume of published toxicologic data.

3.5.2 Materials and Methods

Test organisms. Random-bred weanling mice (21 to 28 days old) are used for Level 1 acute *in vivo* rodent tests. Weanlings are used because they are likely to be more sensitive to toxic effects of test materials than adult mice. In addition, significantly less test material is required for dosing.

Weanling mice may be purchased directly or females with timed pregnancies may be obtained from laboratory breeding colonies or from commercial suppliers. Litters are adjusted to five males and five females shortly after birth to help standardize and enhance pup growth.

Weanlings from random-bred laboratory rats may be used if difficulties are encountered in gavaging weanling mice because of their small size. Rats have been found to be equally as sensitive as mice for this assay.

Test material. Solid materials are generally tested without processing. Samples that are not finely divided may be ground with a mortar and pestle. There are no particle size requirements for this test but material should be fine enough to pass through a 24-gauge gavage needle when in suspension. Solids are solubilized or suspended in deionized water. Primary dosing suspensions are prepared 24 hours in advance to permit water soluble materials to leach into the water at room temperatre. Other vehicles such as corn oil, olive oil or carboxymethyl cellulose may be used if water is not suitable.

Aqueous-liquid samples are concentrated 4- to 10-fold (Section 2.3.8). Nonaqueous liquids are used without preparation. Organic extracts, such as SASS sorbent resin extracts, should be concentrated and solvent exchanged according to Section 2.3.6.

3.5.3 Experimental Design

Since the major objective of the Level 1 biological testing procedure is to identify toxicological problems at minimal cost, it is recommended that a two-step approach be taken to the initial acute *in vivo* toxicological evaluation of unknown compounds. The first is based on the quantal (all-or-none) response and the second on the quantitative (graded) response. Normally, the quantal test is used to determine the necessity for carrying out the quantitative assay. If no animals die following exposure to the in the quantal test, further in vivo testing is not initiated and the sample toxicity is categorized as not detectable (ND).

Quantal. Five male and five female weanling random-bred mice (21 to 28 days old) are used for dosing at the maximum applicable dose (MAD) (5 ml/kg for liquids and 5 gm/kg for solids). The weight of each animal is determined before dosing. The test material is administered by gavage to this population of animals in one, or up to three fractionated doses within eight hours, to give a total dose of 5 ml/kg for liquids or 5 gm/kg for solids (solubilized or suspended in a suitable vehicle). The sample is administered using 1-ml plastic syringes and 24-G gavage needles. The test material should be well mixed when aliquots are removed for dosing. The maximum volume administered to weanling mice should not exceed 0.3 ml at each administration.

Immediately following administration of the test substance and at frequent intervals during the first day, observations of the frequency and severity of all toxic signs or pharmacological effects (Table 3.10) are recorded on an observation checklist (9). Particular attention is paid to time of onset and disappearance of signs. Daily observations are made and recorded on all animals for a 14-day period. At termination of the observation period, all surviving animals are weighed and killed; gross necropsies are then performed. Necropsies are also performed on all animals that die during the course of this study.

Quantitative. If a single animal in the quantal study dies during the 14-day observation period, a quantitative study is performed. Fifty weanling mice or rats, equally divided by sex, are used for this study. After determining good health in the study population following seven days of quarantine, the animals are randomly divided into five groups of ten animals (five of each sex). The test substance is administered in graded dosages according to the following schedule: 1.0, 0.5, 0.1, and 0.05 g/kg or ml/kg. A different dosage schedule may be selected depending on the results of the quantal study. A control group receives an amount of the vehicle equal to the maximum amount of vehicle used in dosing the test material. The observations, study duration, and necropsy procedures are carried out as described for the quantal test.

3.5.4 Results and Data Interpretation

Quantal. If no mortality occurs in the quantal study, no further studies will be performed with the test substance and the LD_{50} should be reported as greater than 5 ml/kg or 5 g/kg. The test material is ranked as having nondetectable toxicity (ND) at the maximum applicable dose (MAD). Effluent samples which produce harmful effects in vivo and do not result in deaths should be noted in the results summary. Such observations are difficult to quantitate but provide insight into the sublethal effects of a sample on rodents. Further investigations may be recommended from observation of nonlethal toxic effects. Initial and final average body weights of the dosed groups are determined and recorded. Gross observations of the necropsies are made and summarized in the report.

TABLE 3.10 DEFINITION OF PHARMACOLOGICAL TOXIC SIGNS

Organ System	Observation and Examination	Common Signs of Toxicity
CNS and somatomotor	Behavior	Change in attitude to observer, unusual vocalization, restlessness, sedation
	Movements	Twitch, tremor, ataxia, catatonia, paralysis, convulsion, forced movements
	Reactivity to various stimuli	Irritability, passivity, anaesthesis, hyperaesthesis
	Cerebral and spinal	Sluggishness, absence of reflexes
	Muscle tone	Rigidity, flaccidity
Autonomic nervous system	Pupil size	Myosis, mydriasis
	Secretion	Salivation, lacrimation
Respiratory	Nostrils	Discharge
	Character and rate of breathing	Bradypnoea, dyspnoea, Cheyne-Stokes respirations, Kussmaul breathing
Cardiovascular	Palpation of cardiac region	Thrill, bradycardia, arrhythmia, stronger or weaker beat
Gastrointestinal	Events	Diarrhea, constipation,
	Abdominal shape	Flatulence, contraction
	Feces consistency and color	Unformed, black or clay colored
	Perianal region	Soiled
Skin and fur	Color, turgor, integrity	Reddening, flaccid skinfold, eruptions, piloerection
Mucous membranes	Conjunctiva, mouth	Discharge, congestion, hemorrhage, cyanosis, jaundice
Eye	Eyeball	Exophthalmus, nystagmus
	Transparency	Opacities
Others	Rectal or paw skin	Subnormal, increased temperature
	General Condition	Abnormal posture, emaciation

Quantitative. The LD_{50} will be calculated by the method of Litchfield and Wilcoxin (20) or other suitable method (e.g. PROBIT) for LD_{50} determination. If the data are not suitable for calculation of a precise LD_{50}, i.e., total mortality occurs for the lowest dose, an estimate of the LD_{50} could be made or the LD_{50} could be expressed as less than 0.05 ml/kg or 0.05 g/kg. Occasionally, it may be necessary to use higher dosages, lower dosages or another series of intermediate dosages depending on the initial results.

The calculated LD_{50} value is used to rank the test sample. Evaluation criteria for toxicity categorization are presented in Table 3.11.

Observations are made and recorded daily on all animals for the 14-day period. As in the quantal phase, no attempt is made to quantitate or rank the observations. The average animal body weight of each group is determined initially and at the termination of the experiment. The average weights, and the weights as fractions of the control are reported for each dose level. Necropsy observations are recorded and reported.

TABLE 3.11 ACUTE *IN VIVO* RODENT ASSAY EVALUATION CRITERIA

Toxicity	Solids (LD_{50} in g/kg)	Liquids (LD_{50} in ml/kg)
High	<0.05	<0.05
Moderate	0.05 to 0.5	0.05 to 0.5
Low	0.5 to 5	0.05 to 5
Not Detectable	>5	>5

CHAPTER 4

LEVEL 1 AQUATIC ECOLOGICAL ASSAYS

4.1. INTRODUCTION AND RATIONALE

Biological responses and bioaccumulation must be considered, as well as chemical and physical parameters, when assessing the potential impact of complex wastes on the aquatic environment. Biological testing for aquatic ecological effects usually consists of static acute toxicity tests on selected organisms representative of the various trophic levels. Evaluation of the bioaccumulation of components in complex mixtures is accomplished using a laboratory technique for simulating bioaccumulation phenomena.

Acute toxicity tests are used to determine the concentration of test material that produces an adverse effect on a specified percentage of the test organisms in a short period of time. Because mortality is normally an easily detected and an obviously important adverse effect, the most common acute toxicity test is the acute lethality test. The index most often used with fish is the 96-hour median lethal concentration (96-hour LC_{50}) and for macroinvertebrates the 48-hour effective concentration (48-hour EC_{50}). The LC_{50} is a statistically derived estimate of the concentration of toxicant in dilution water that is lethal to 50 percent of the test organisms during continuous exposure for a specified period of time, based on data from one experiment. This may be supplemented, in fish tests with effects on behavior. The EC_{50} for macroinvertebrates is an estimate of the concentration of test material that results in the immobilization of 50 percent of the test organisms during continuous exposure for a specified period of time in one experiment. Immobilization is defined as lack of movement except for minor activity of appendages. This measured effect is used because death is not always easily determined with some invertebrates.

In algal tests the principle criterion of toxicity is the effect on growth during continuous exposure for a specified period of time. The exposure period for the freshwater algal bioassay is 120 hours, while that for the marine algal bioassay is 96 hours. The 96- or 120-hour effective concentration (EC_{50}), the concentration in which algal growth is inhibited by 50 percent as compared with growth in the control, is statistically estimated. For samples which stimulate algal growth, the stimulatory concentration (SC_{20}) is calculated. The 96- or 120-hour SC_{20} is defined as the concentration causing a stimulation in growth of 20 percent relative to the control after 96 or 120 hours of exposure. Other related criteria which may be useful are the effects on rates of growth, on maximum standing crops, and on algal biomass at the end of the assay.

It may also be possible to establish the approximate concentration of test material which produces no observable deleterious effect by any of the criteria under study, which is the No Observed Effect Concentration (NOEC).

Since the reporting for each test is unique, guidelines are given for individual tests in each section and summarized in Table 4.11.

The recommended test organisms in freshwater tests are the algae *Selenastrum capricornutum*, juvenile fathead minnow *Pimephales promelas*, and early instars of *Daphnia magna*. The recommended test period is 120 hours for the algal test, 96 hours for the fish study, and 48 hours for the daphnid study. Thus, the principal finding obtained from an algal study is the 120-hour EC_{50} or SC_{20}, from the fish study the 96-hour LC_{50} and from the daphnid study the 48-hour EC_{50}. Table 4.1 describes the biological characteristics of the aquatic bioassays.

The suggested test organisms in marine tests are the algae *Skeletonema, costatum*, the juvenile sheepshead minnow *Cyprinodon variegatus*, and the adult mysid *Mysidopsis bahia*. The primary parameters of toxicity obtained from a marine algal study are the 96-hour EC_{50}, or SC_{20}, from the marine fish study the 96-hour LC_{50} and from the mysid study the 96-hour EC_{50}. The characteristics of the marine aquatic tests are also described in Table 4.1.

The aquatic tests described in this section are well suited for Level 1 environmental assessment testing because they develop useful information quickly and at low cost. The six recommended aquatic bioassays have been routinely used by EPA and others to monitor the biological impact of effluents on the environment. There already exists a body of published material and technical expertise which can assist in the application and interpretation of Level 1 aquatic testing. These tests measure the effect of a test material on organisms that represent three successively higher trophic levels characteristic of either fresh or marine waters. Selection of the freshwater or marine battery of tests is made based on the type of receiving water into which the effluent is discharged. Their principal limitations are (1) that they usually do not closely simulate the characteristics of the receiving waters into which the test effluent is actually being discharged, and (2) that the species tested may not be representative of the most sensitive species native to those waters. They do, however, make it possible to rank municipal and/or industrial effluents in order of relative toxicity.

The procedures for the aquatic ecological assays have been developed largely from References 21 and 22. Modifications to the original protocols have been made where necessary to adapt tests to the requirements of Level 1 environmental assessment.

4.1.1 General Materials and Methods For Aquatic Ecological Assays

Materials and methods that are common to all, or nearly all, Level 1 aquatic ecological tests are presented in this section. The section for each specific test discusses materials and methods unique for that test and identifies which of the general materials and methods are applicable.

TABLE 4.1

CHARACTERISTICS OF LEVEL 1 AQUATIC ECOLOGICAL EFFECTS BIOASSAYS

Characteristic	Static Acute Aquatic Bioassay	Algal Bioassay
Freshwater Species	Fish - Fathead Minnow, Invertebrate - *Daphnia*	*Selenastrum*
Marine Species	Fish - Sheepshead Minnow, Invertebrate - *Mysidopsis*	*Skeletonema*
End Point(s) Measured	Lethality	Cell Population Growth
Amenable to Sample Types	Liquids, Solids (leachates)	Liquids, Solids (Leachates)
Data Expression	LC_{50}	EC_{50}, SC_{20}
Special Features	Can detect whole-animal effects on key aquatic ecological consumers	Effective measure of toxicity to aquatic producers

Facilities. The facilities should include tanks equipped for temperature control and aeration for holding and acclimating test organisms, as well as a constant temperature area or recirculating water bath for the test vessels. If the use of reconstituted dilution water is necessary, there should be a tank for its preparation. If air is used for aeration, it must be free of oil and fumes. The test facility must be well ventilated and free of fumes. Illumination should be provided of an intensity and duration that is specified in the Materials and Methods section for each test.

Construction materials. Materials that come in contact with effluent samples, stock solutions or test solutions should minimize sorption of any constituents of the test material and not contain any substances that can be leached or dissolved by the water. Glass, #316 stainless steel, and perfluorocarbon plastics must be used whenever possible to minimize leaching, dissolution and sorption. Unplasticized plastics may be used for holding and acclimation tanks and in the water supply system. Rubber, copper, brass, and lead should be avoided. If stainless steel is used it must be welded, never soldered. Silicone adhesive used to cement glass containers sorbs some organochlorine and organophosphorus compounds which are difficult to remove; therefore, as little adhesive as possible should be in contact with test-material solutions and extra beads of adhesive should be on the outside, not the inside, of the containers.

Test containers. Fish tests should be conducted in 19.6-liter wide-mouth soft-glass jars or in all-glass containers 30 cm wide, 60 cm long and 30 cm high. Daphnids should be exposed in 3.9-liter wide-mouth soft-glass bottles, in 3.3-liter battery jars or in 250-milliliter beakers. Mysid tests are conducted in 2-liter culture dishes containing 1 liter of test medium. Freshwater and marine algal tests should be conducted in Erlenmeyer culture flasks of Pyrex or Kimax type of glass. The flask size is not critical, but because of CO_2 limitations, the volume-to-volume ratio is. The recommended contents-to-flask-volume ratios for hand-shaken flasks are:

25 ml in 125 ml flask
50 ml in 250 ml flask
100 ml in 500 ml flask

Maximum permissible contents-to-volume ratios in continously shaken flasks should not exceed 50 percent.

Cleaning and preparation of glassware. Each container for fish or macroinvertebrate testing must be cleaned before use. A new container must be (1) washed with non-phosphate detergent, (2) rinsed with 100 percent acetone, (3) rinsed with water, (4) rinsed with 5 percent nitric acid and (5) rinsed thoroughly with tap or other clean water. After testing, each container should be (1) emptied, (2) rinsed with water, (3) cleaned with a material suitable for removing the toxicant tested (such as acid to remove metals and bases and solvent to remove organic compounds) and (4) rinsed thoroughly with water. Dilute acid is also used to remove mineral deposits. Containers should be disinfected for one hour with an iodophor, 200 mg hypochlorite per liter or a quaternary ammonium salt such as 800 ppm Roccal II* with at least one thorough scrubbing during the hour, then rinsed thoroughly. For safety, acid and hypochlorite should not be used together.

All glassware used in freshwater or marine algal testing is prepared as above with the following exception. The final rinse should be of deionized water filtered through a 0.22 μm membrane filter if the Coulter Counter is to be used in freshwater algal assays. Flasks are dried in an oven at 50° to 70°C. Foam plugs are inserted and the glassware is autoclaved for 20 minutes at 1.1 kg/cm^2 (15 psi) and 121°C. Cooled flasks are stored in closed cabinets.

Receipt and quarantine for fish. Stock fish shipped from outside sources may have been subjected to changes in temperature, dissolved oxygen and pH, handling disturbances and other stresses, and should be examined carefully for health and vigor. Holding water should be introduced gradually into the shipping bags, and fish observed for abnormal behavior.

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*National Laboratories, Montvale, NJ 07645

When the difference in water temperature between the bag and holding tank is 2°C or less, fish from one bag should be introduced into the tank and observed for acute stress. If acute stress is not seen, the remaining fish may be introduced into the tank in a similar manner.

To prevent spread of disease, incoming fish for stock should be quarantined for at least two weeks and observed for abnormal behavior and parasites. The quarantine tanks should be prepared in advance by thorough scrubbing and cleaning with an industrial cleaner, rinsing with water, sterilizing with a quaternary ammonium salt such as 800 ppm Roccal II, and rinsing with at least three changes of water before filling with dilution water. If after two-weeks' quarantine they show no signs of infection or abnormal behavior they are transferred to stock-holding tanks, otherwise, they are either discarded or treated as described in Disease treatment for fish.

To prevent initiation and spread of disease, nets, buckets, fish graders and hands should be routinely disinfected with 200 ppm Roccal II before being placed in the water.

Disease treatment for fish. Freshwater fish may be chemically treated to cure or prevent diseases by using the treatments recommended in Table 4.2. Some of the treatments (formalin and potassium permanganate) listed in Table 4.2 may also be used to cure or prevent diseases in marine fishes. However, if a group of either type of fish is severely diseased, the entire lot should be destroyed. Generally, the fish should not be treated during the first 16 hours after arrival at the facility because they may be stressed because of collection or transporation and some may have been treated just prior to transit. Tests must not begin with treated fish for at least four days after treatment. Tanks and test chambers which may be contaminated with undesirable microorganisms should be disinfected following the procedures outlined in Cleaning, in this section.

Test material. The test material may be a solid, aqueous liquid, or nonaqueous liquid. Quantity of sample required, is listed in Table 4.3. Samples are usually tested directly without preparation, however, some test materials require pretest preparation. Table 2.4 lists pretest preparation requirements for individual sample types and Section 2.3 details specific procedures. Aqueous effluents and aqueous leachates of solid samples should be run directly and must not be aerated or altered in any way, except that aqueous effluents may be filtered through a sieve or screen with holes 2 mm or larger to remove large particles. Nonaqueous samples are diluted in the appropriate solvent with uniform aliquots added to each test container or are added directly by volume and diluted with dilution water. If possible, samples should be solvent exchanged to a solvent compatible with the test organisms. The maximum applicable dose is determined uniquely for each nonaqueous liquid sample based upon the solubility of the sample in water and the toxicity of solvents used to test organisms. Samples must be covered at all times and violent agitation must be avoided. Undissolved materials must be uniformly dispersed by gentle agitation immediately before a portion of the sample is removed for use. In handling samples containing highly volatile substances, it may be desirable to add the test sample below the surface of the dilution water.

TABLE 4.2 RECOMMENDED PROPHYLACTIC AND THERAPEUTIC TREATMENTS FOR FRESHWATER FISH[a]

Disease	Chemical	Conc., mg/l	Application
External bacteria	Benzalkonium chloride (Hyamine 1622®)	1-2 AI[b]	30-60 min[c]
	Nitrofurazone (water mix)	3-5 AI	30-60 min[c]
	Neomycin sulfate	25	30-60 min[c]
	Oxytetracycline hydrochloride (water soluble)	25 AI	30-60 min[c]
Monogenetic trematodes, fungi and external protozoa[d]	Formalin plus zinc-free malachite green oxalate	25 0.1	1-2 hours[c]
	Formalin	150-250	30-60 min[c]
	Potassium permanganate	2-6	30-60 min[c]
	Sodium chloride	15,000-30,000 2000-4000	5-10 min dip [c,e]
	Dexon® (35% AI)	20	30-60 min[c]
Parasitic copepods	Trichlorfon (Masolen®)	0.25 AI	[f]

[a] These recommendations do not imply that these treatments have been cleared or registered for these uses. Appropriate State and Federal regulatory agencies should be consulted to determine if the treatment in question can be used and under what conditions the uses are permitted. These treatments should be used only on fish intended for research. They have been found dependable, but efficacy against diseases and toxicity to fish may be altered by temperature or water quality. Researchers are cautioned to test treatments on small lots of fish before making large-scale applications. Prevention of disease is preferred, and newly acquired fish should be treated with the formalin-malachite green combination on three alternate days if possible. However, in general, fish should not be treated on the first day they are in the facility. This table is merely an attempt to indicate the order of preference of treatments that have been reported to be effective. Before a treatment is used, additional information should be obtained from such sources as Davis (23), Hoffman and Meyer (24), Reichenbach-Klinke and Elkan (25), Snieszko (26) and van Duijn (27).

[b] AI - active ingredient.

[c] Treatment may be accomplished by (1) transferring the fish to a static treatment tank and back to a holding tank; (2) temporarily stopping the flow in a flow-through system, treating the fish in a static manner and resuming the flow to flush out the chemical or (3) continuously adding a stock solution of the chemical to a flow-through system by means of a metered flow or the technique of Mount and Brungs (28).

[d] One treatment is usually sufficient except for "Ich", which must be treated daily or every other day until no sign of the protozoan remains. This may take 4 to 5 weeks at 5 to 10°C and 11 to 13 days at 15 to 21°C. A temperature of 32°C is lethal to Ich in 1 week.

[e] Minimum of 24 hours, but may be continued indefinitely.

[f] Continuous treatment should be employed in static or flow-through systems until no copepods remain, except that treatment should not be continued for over 4 weeks and should not be used above 27°C.

TABLE 4.3 SAMPLE SIZE REQUIREMENTS FOR AQUATIC ECOLOGICAL ASSAYS

Type of Test	Solid (kilograms)	Liquid Aqueous (liters)	 Nonaqueous[a] (milliliters)
Freshwater Fish	10 (7.5)[b]	40 (30)	1000 (750)[c]
Freshwater Invertebrate	0.5 (0.3)	2 (1.5)	200 (150)
Freshwater Algae	0.25 (0.13)	1 (0.6)	100 (60)
Marine Fish	10 (7.5)	40 (30)	1000 (750)
Marine Invertebrate	2 (1.2)	8 (6)	800 (600)
Marine Algae	0.25 (0.13)	1 (0.6)	100 (60)

[a] Nonaqueous liquids include aqueous samples with greater than 0.2% organics, nonaqueous liquids, solvent exchange samples, and extracts or leachates in a nonaqueous (organic) vehicle.

[b] The first value given is the requested sample size for routine Level 1 testing. The value in parentheses is the minimum feasable sample size to conduct the test.

[c] The maximum applicable dose (MAD) and the volume of nonaqueous liquid samples required for aquatic ecological testing is dependent upon the solubility of the sample in water. The MAD is determined for each sample before testing is initiated. For additional information, contact the Technical Support Staff, Process Measurements Branch, IERL-RTP, U.S. EPA, Research Triangle Park, NC 27711.

If testing is to be done on-site, the tests should begin within eight hours of collection. If testing is to be done at a laboratory, the samples should be placed on ice for preservation during the transportation and testing performed as soon as possible after laboratory receipt of the samples. Samples should be stored at 4°C if testing is not initiated upon sample receipt. The temperature of the sample should be adjusted to that of the test (± 2°C) before portions are added to the dilution water. Solid materials may be added directly to dilution water.

Dissolved oxygen concentration. Aeration of test solutions during the test should be avoided to minimize loss of highly volatile materials. It should be noted in the final report if the dissolved oxygen concentration is less than 40 percent saturation in any test chamber for freshwater fish or Daphnia tests, or less than 60 percent for marine fish or mysid tests. Neither freshwater nor marine algal tests have defined dissolved oxygen concentration standards.

4.2 STATIC ACUTE TOXICITY TESTS WITH FRESHWATER FISH AND DAPHNIA

4.2.1 Introduction and Rationale

The static toxicity tests with freshwater fish and Daphnia provide a large amount of data in a short period of time. The recommended test organisms are the juvenile fathead minnow, *Pimephales promelas*, and early instars of *Daphnia magna*. The static acute exposure period for the fathead minnow is 96 hours and 48 hours for the daphnid study. The 96-hour mean lethal concentration (96-hour LC_{50}) is calculated for the fathead minnow. Because death is not always easily determined in Daphnia, the 48-hour effective concentration (48-hour EC_{50}) is calculated for Daphnia.

4.2.2 Materials and Methods

General procedures listed for all aquatic tests in section 4.1.1 are applicable to the static acute toxicity tests with freshwater fish and Daphnia. Specific areas discussed in Section 4.1.1 that should receive careful attention are: facilities, construction materials, test containers, cleaning and preparation of glassware, receipt and quarantine for fish, disease treatment for fish, test material and dissolved oxygen concentration. Materials and methods unique to freshwater fish and Daphnia tests are included below.

Dilution water. A minimal criterion for acceptable dilution water is that healthy organisms will survive in it for the duration of acclimation and testing without showing signs of stress such as discoloration or unusual behavior. Water in which daphnids will survive and reproduce satisfactorily should be an acceptable dilution water for tests with freshwater organisms.

The dilution water should be of constant quality and analyzed by the methods given in References 29, 30, 31, and 32 to ascertain that none of the following substances exceeds the maximum allowable concentration shown:

Pollutants	Maximum Concentration
Suspended solids	20 mg/l
Total organic carbon	10 mg/l
Un-ionized ammonia	20 μg/l
Residual chlorine	3 μg/l
Total organophosphorus pesticides	50 ng/l
Total organochlorine pesticides plus PCB's	50 ng/l

The dilution water is considered to be of constant quality if the monthly ranges of hardness, alkalinity and conductivity are within 10 percent of their respective means and if the monthly range of pH is less than 0.4 units. Reconstituted dilution water may be prepared according to the method of Marking and Dawson (33). For comparability of results between tests, hardness should be as close as possible to 100 mg/l as $CaCO_3$.

4.2.3 Test Organisms

Species. The juvenile fathead minnow, *Pimephales promelas*, and early instars of *Daphnia magna* are the species to be used in Level 1 freshwater static acute toxicity tests. The fathead minnow is a warm-water fish of ponds, lakes and sluggish streams. Daphnids occur in nearly all types of freshwater habitats. Both species, because of their wide geographic distribution, important places in the aquatic food web, temperature requirements, wide pH tolerance, ready availability and ease of culture, have been recommended as bioassay organisms by the Committee on Methods for Toxicity Tests with Aquatic Organisms (21).

Source. Fathead minnows may be obtained from private, State, or Federal fish hatcheries, or captured from wild populations in relatively unpolluted areas. However, collecting permits may be required by local and State agencies. Fish collected by electroshocking should not be used. *Daphnia* should be reared in the testing facility from laboratory cultures.

Sizes, life stages. Fathead minnows used in testing should weigh between 0.5 and 1.0 g each. All fish in each test should be from the same year class, and the standard length (tip of snout to end of caudal peduncle) of the longest fish should be no more than twice that of the shortest fish. Weights and lengths should be determined by measuring representative specimens before the test or control fish after the test. Very young fish (not yet actively feeding), spawning fish and spent fish should not be used.

Daphnia magna used in testing should be in the early instar stages of their life cycle. All organisms in a test must be from the same source and as healthy and uniform in size and age as possible.

Culturing, care and handling. Fathead minnows are maintained at 20-22°C in a flow-through system with a turnover of at least two volumes daily, or in a recirculating system in which the water is passed through a carbon filter and an untraviolet sterilizer.

Daphnia magna are maintained in a static system at 19-22°C. Tanks must be siphoned periodically to remove debris and water added as necessary to maintain volume. Cultures must be maintained under optimum conditions at all times to prevent formation of ephippial eggs; daphnids from cultures in which ephippia are being produced must not be used in testing.

Both species should be fed at least once a day, at which time careful observations should also be made for mortality and for signs of disease, stress and injury. Fish are fed a commercial fish food such as Purina Trout Chow*. *Daphnia* are fed 1.25 mg (dry weight) of a mixed freshwater algal culture per liter of water daily. Dead and abnormal fish or *Daphnia* should be removed as soon as they are observed.

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*Ralson Purina Co., St. Louis, MO 63188.

Water quality should be held constant as described earlier and temperature changes should not exceed 3°C in any 12-hour period. Fish tanks should be scrubbed at least twice a week.

The organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully and quickly as possible so that the organisms are not needlessly stressed. Small dip nets are best for handling fish and wide-bore pipettes for Daphnia. Organisms that touch dry surfaces or are dropped or injured during handling should be discarded.

From the time test organisms are first cultured or received they should be shielded from disturbances; overcrowding should be avoided.

Holding and acclimation. After collection or transportation, the fish or Daphnia should be held in and acclimated to the dilution water for at least two days before beginning a test under the same holding conditions as described earlier in Care and handling.

A group of animals must not be used for a test if individuals appear to be diseased or otherwise stressed or if more than 5 percent die within 48 hours prior to beginning the test. If a group fails to meet these criteria, it must be discarded or treated and held an additional 4 days.

Fathead minnows should not be fed for 48 hours prior to the beginning of a test. However, the Daphnia may be fed up to the beginning of the test.

4.2.4 Experimental Design

Test procedure. Unless the approximate toxicity of the sample is already known, at least six concentrations of test material should be prepared. The highest dose should be at the maximum applicable dose (MAD) for that sample type (Table 4.11) unless physical characteristics of the sample or other previously gathered toxicity data contravene this. The concentrations should be in a geometric series; each one should be at least 50 percent of the next higher one.

In fathead minnow tests, at least 10 fish must be exposed to each test concentration per replicate with two replicates per concentration used in the test. For Daphnia magna tests, 10 organisms per replicate with three replicates per concentration should be used. The use of more organisms and replicate test containers and random assignments of test organisms to containers is desirable.

The fathead minnow tests should be conducted at 22 ± 2°C, and those with Daphnia at 19 ± 2°C. A photo period of 16 hours light and 8 hours dark is used for both tests. Neither type of test animal should be fed during exposure. The test conditions are summarized in Table 4.4.

TABLE 4.4 SUMMARY OF TEST CONDITIONS, FRESHWATER FISH OR MACROINVERTEBRATE TEST

	Fathead Minnow, Pimephales promelas	Daphnia magna
Temperature, °C	22 ± 2	19 ± 2
Photoperiod, hours light:dark	16:8	16:8
Water quality, hardness mg/l as $CaCO_3$	100	100
Container size	19.6 liters	250 ml
Test volume	15 liters	200 ml
Organisms per container	10	10
Replicates	2	3
Feed	No	No
Duration, hours	96	48
Measurements of D.O. and pH, hours	0, 24, 48, 72, 96	0, 48

Each test requires a control which consists of the same dilution water, conditions, procedures and organisms as used in the test concentrations. If any solvent other than water is present in the test concentration, a solvent control is also required. This solvent control is treated the same as the control except that the amount of solvent used in dosing the test containers is added to this solvent control.

In the fathead minnow test there should be 15 liters of test solution or control water in each 19.6-liter jar. If 30 x 30 x 60 centimeter containers are used, the solution should be between 15 and 20 centimeters deep.

In the daphnid test there should be 2 to 3 liters of solution or control water in each 3.9-liter wide-mouth bottle or 3.3-liter battery jar, or 200 milliliters in each 250-milliliter beaker.

Test organisms should be placed in the test and control vessels not more than 30 minutes after the test solutions are prepared. Ten fish in each vessel and 10 daphnids in each replicate are recommended. Chemical, physical and biological data are taken and recorded as described in

Section 4.2.5 for the duration of the test (96 hours for the fathead minnow, 48 hours for the daphnid test.

If no toxicity is detected at any concentration and the MAD dose was tested, then no further testing is required. The test material may be reported as having no detectable toxicity. Test materials that kill or immobilize all or nearly all the test organisms should be retested at a lower dose range.

Biological loading. The biological loading in each test and control vessel should not exceed 0.8 g of test organism per liter or be so high as to (1) reduce dissolved oxygen concentration below 40 percent saturation, (2) raise the concentration of metabolic products above acceptable levels or (3) stress the organisms by overcrowding, any of which may invalidate the test results.

4.2.5 Results and Data Interpretation

Chemical and physical data. In the fathead minnow test, dissolved oxygen concentration, and pH should be measured at the beginning of the test and every 24 hours thereafter in the controls and in the high, medium and low concentrations. Conductivity should be measured at the beginning of the test in the control and each test concentration. Temperature of the water bath or controlled-temperature area should be recorded continuously or every 24 hours.

In the *Daphnia* test, dissolved oxygen, pH, and conductivity (when required) on the high, medium and low concentrations, and temperature should be recorded initially and at 48 hours.

Concentration of un-ionized ammonia, if required, can be obtained by measuring total ammonia and consulting Reference 30.

Biological data. Mortality is the effect most often used to define acute toxicity to aquatic organisms. Criteria for death are usually lack of movement, especially of gill movement in fish, and lack of reaction to gentle prodding.

Because death is not always easily determined with some invertebrates, an EC_{50} may be calculated rather than an LC_{50}. The principal criterion for effect on *Daphnia* is immobilization, defined as lack of movement except for minor activity of appendages.

Mortality or immobilization and abnormal behavior should be recorded every 24 hours for the duration of the test. Observations of test materials which produce harmful effects *in vivo*, but do not result in deaths, are difficult to quantitate. Such observations provide insight into the sublethal effects of a sample on aquatic organisms and may be used to recommend further investigation of the test material. Dead or immobilized organisms should be removed as soon as they are observed. Table 4.5 lists definitions of fish behavior terms, and suggested code for recording and reporting. If more than 10 percent of test organisms in any control die or are immobilized, the entire test is unacceptable.

TABLE 4.5 DEFINITION OF FISH BEHAVIOR TERMS

Code	Term	Definition
1.	General Behavior.	Observable responses of the test fish, individually or in groups, to the range of factors constituting their environment.
a.	Quiescent:	marked by a state of inactivity or abnormally low activity; motionless or nearly so.
b.	Hyperexcitable:	reacting to stimuli with substantially greater intensity than control fish.
c.	Irritated:	exhibiting more or less continuous hyperactivity.
d.	Surfacing:	rising and remaining unusually long at the surface.
e.	Sounding:	diving suddenly straight to the bottom; remaining unusually long at the bottom.
f.	Twitching:	moving the body or parts of the body with sudden jerky movements.
g.	Tetanous:	in a state of tetany; marked by intermittent tonic spasms of the voluntary muscles.
h.	Flaccid:	lacking tone, resilience or firmness; weak and enfeebled; flabby.
i.	Normal:	unaffected by or not exposed to a particular experimental treatment; conforming to the usual behavioral characteristics of the species.
2.	Swimming.	Progressive self-propulsion in water by coordinated movement of tail, body, fins.
a.	Ceased:	Broken off or tapered off to a stop.
b.	Erratic:	Characterized by lack of consistency, regularity or uniformity; fluctuating, uneven; eccentric.
c.	Gyrating:	Revolving around a central point; moving spirally about an axis.
d.	Skittering:	skimming hurriedly along the surface with rapid body movements.
e.	Inverted:	turned upside down, or approximately so.
f.	On side:	turned 90° laterally, more or less, from the normal body orientation.
3.	Pigmentation.	Color of skin due to deposition or distribution of pigment.
a.	Light discolored:	color appearance lighter than usual for the species.
b.	Dark discolored:	color appearance darker than usual for the species.
c.	Varidiscolored:	color appearance abnormally varied; mottled.
4.	Integument.	The skin.
a.	Mucus shedding:	observably losing mucous skin coating to an abnormal degree.
b.	Mucus coagulation:	showing observable clumping or clotting of the mucous skin coating, especially at the gills.
c.	Hemorrhagic:	visibly bleeding as from gills, eyes, anal opening.
5.	Respiration.	Physical action of pumping water into mouth and out through gills, so as to absorb oxygen.
a.	Rapid:	observably faster than normal to a significant degree.
b.	Slow:	observably slower than normal to a significant degree.
c.	Irregular:	failing to occur at regular or normal intervals.
d.	Ceased:	broken off or tapered off to a stop.
e.	Gulping air:	swimming at surface with mouth open and laboriously pumping surface water and air through gills.
f.	Labored:	performed with apparently abnormally great difficulty and effort.
	No Observed Effect Concentration:	The highest test concentration in which fish experience no mortality and exhibit no observable behavioral abnormalities at any time during a specified period of exposure to the test material. Ordinarily determined for periods from the start of testing to the end of each successive 24 hours.

Laboratory data forms. Forms for recording chemical, physical and biological data in both fathead minnow and *Daphnia* tests are illustrated in Quality Control documents (9).

Calculations. The concentration of test material lethal to 50 percent of the population (LC_{50}) and 95 percent confidence limits should be determined at 24-, 48-, 72-, and 96-hour exposures for fish tests, and the EC_{50} and 95 percent confidence limits at 24- and 48-hour exposures for *Daphnia magna* tests. Any of several methods including moving average, Spearman Kärber, Litchfield-Wilcoxin, probit or binomial may be used. For a discussion these methods, refer to the review article by Stephan (34). The results (96 hours for fish and 48 hours for *Daphnia*) are evaluated according to Table 4.11 which defines the toxicity categories.

4.3 FRESHWATER ALGAE 120-HOUR SCREENING TEST

4.3.1 Introduction and Rationale

Unicellular algae are important producers of oxygen and form the basis of the food web in aquatic ecosystems. Since algal species and communities are sensitive to environmental changes, growth may be inhibited or stimulated by the presence of pollutants. Therefore, the response of algae must be considered when assessing the potential ecological effects of industrial or municipal discharges on aquatic ecosystems.

A simple screening test for toxicity to algae can be conducted in 120 hours. Algae are exposed to various concentrations of the test material; growth is measured at 120 hours. Results are expressed in terms of the EC_{50} (the lowest test concentration causing inhibition of growth by 50 percent relative to the control) and the no observed effect concentration (NOEC, the highest test concentration in which growth is not significantly different from that in the control). Stimulatory effects, if any, should be noted and expressed mathematically in terms of SC_{20} and used for estimation of bioactivity of effluent.

4.3.2 Materials and Methods

General procedures listed for all aquatic tests in Section 4.1.1 are applicable to the static acute toxicity test with freshwater algae. Specific areas discussed in Section 4.1.1. that should receive careful attention are facilities, construction materials, test containers, cleaning and preparation of glassware, and test material.

Materials and methods unique to freshwater algal tests follow.

Equipment. Equipment should include a constant-temperature room or incubator capable of providing temperature control of 24 ± 2°C. A gyrotary shaking apparatus capable of 100 oscillations per minute should be available for test culture flasks. Continuous illumination of 4300 ± 650 lumens/ m^2 (400 ft-c) is required for freshwater green algae. Overhead cool-white fluorescent bulbs should be used. Light intensity is measured adjacent to the flask at liquid level using a light meter capable of being calibrated against National Bureau of Standards lamps.

Culture containers for this and other aquatic tests are discussed in Section 4.1.1. Erlenmeyer culture flasks of Pyrex or Kimax glass are used in either 125-, 250-, or 500-ml sizes. The recommended contents-to-volume ratio in hand-shaken flasks is 1 to 5 and should not exceed 50 percent for continuously shaken flasks (35). Flask closures must permit gas exchange and prevent contamination. Foam plugs* are suggested. Since some brands may be toxic, each laboratory must determine for each type of closures purchased whether or not there are any significant effects on algal growth.

Support equipment includes an autoclave or pressure cooker capable of producing 1.1 kg/cm^2 (15 psi) at 121°C and a dry-heat oven capable of a temperature of 120 ± 1°C. A Coulter Counter with a mean-cell-volume computer (MCV/MHR) or high-quality microscope is needed for biomass measurements.

Freshwater Algal Nutrient Medium. Algal Assay Medium (AAM) is prepared in the order listed in Table 4.6 by adding 1.0 ml of each of the macronutrient stock solutions, to 900-ml deionized water, with mixing after each addition. Then 1.0 ml of the micronutrient stock is added and the final volume brought to 1 liter with deionized water. The mixture is filter-sterilized by passing through a 0.22 μm porosity membrane filter (pre-rinsed with 100-ml deionized water) into a sterile container.

Medium is stored in the dark at 4°C to reduce possible photochemical changes and bacterial growth.

4.3.3. Test Organisms

For freshwater algal assays, the recommended test organism is *Selenastrum capricornutum*, a unicellular non-motile chlorophyte that is easily maintained in laboratory cultures.†

Stock cultures of algae should be maintained at 24°C ± 2°C under continuous illumination in AAM. It is recommended that several cultures be maintained on agar under axenic conditions. Transfers of stock cultures should be made every six to eight days to provide cultures with a sufficient number of cells growing exponentially for test inoculations. All transfers must follow standard microbiological techniques to ensure a minimum of contamination. Prior to establishing an algal culture, the culture techniques of Miller *et al.*, should be reviewed (35).

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*For example, Gaymar Industries, Inc., Orchard Park, NY 14127.

†Cultures can be obtained from Joseph C. Green, Environmental Reseach Laboratory, U.S. EPA, Corvallis, Oregon 97330.

TABLE 4.6 COMPOSITION OF ALGAL ASSAY MEDIUM (AAM)

Macronutrients

Stock Solutions[a]		Nutrient Composition Prepared Medium	
Compound	Concentration (g/l)	Element	Concentration (mg/l)
$NaNO_3$	25.500	N	4.200
$NaHCO_3$	15.000	Na	11.001
		C	2.143
K_2HPO_4	1.044	K	0.469
		P	0.186
$MgSO_4 \cdot 7H_2O$	14.700	S	1.911
$MgCl_2 \cdot 6H_2O$	12.164	Mg	2.904
$CaCl_2 \cdot 2H_2O$	4.410	Ca	1.202

Micronutrients

Stock Solutions[a]		Nutrient Composition Prepared Medium	
Compound	Concentration (mg/l)	Element	Concentration (µg/l)
H_2BO_3	185.520	B	32.460
$MnCl_2 \cdot 4H_2O$	415.610	Mn	115.374
$ZnCl_2$	3.271	Zn	1.570
$CoCl_2 \cdot 6H_2O$	1.428	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.260	Mo	2.878
$FeCl_3 \cdot 6H_2O$	160.000	Fe	33.051
$Na_2EDTA \cdot 2H_2O$	222.000	--	--

[a] Other forms of the salts may be used as long as the resulting concentrations of elements are the same.

4.3.4. Experimental Design

Preparation of Toxicant. Depending on its nature, the test material is prepared by one of two methods. In the first method, solids or non-aqueous liquid materials may be added directly by weight or volume respectively to the algal medium or a concentrated stock solution may be prepared in deionized water (or a solvent such as ethanol, acetone, dimethylforamide or triethyleneglycol) and equal volume aliquots of a small size are added to each treatment. If it is not possible to prepare a homogenous solution of the toxicant, it must be added directly into each replicate flask.

The second method, for aqueous effluents or aqueous leachates of solids, allows testing by percent volume (volume/volume). Nutrients are added to one liter of effluent in the same quantities as in the control algal assay medium. The effluent is used up to 80 percent volume-per-volume in the test. Additional test concentrations are prepared on a volume-percent basis by mixing appropriate volumes of effluent with control medium.

Test Procedure. Six test concentrations and a negative control are normally tested, with four replicates of each. Three replicates are inoculated with algae while the fourth serves as a blank. Three replicates are necessary for statistical analyses and the blank is necessary to correct biomass measurements for particulates which may be present in the test treatments.

If a solvent is necessary for the preparation of the test material, a solvent control must be included. All flasks and the solvent control must contain the same amount of solvent. The toxicity of commonly used solvents should be determined in each laboratory using this bioassay to help select suitable solvent levels.

1. Inoculum. A 6- to 8-day-old stock culture is used as the inoculum source. Population density in the stock culture is determined by direct counting or spectrophotometry. A volume of inoculum calculated to yield an initial concentration of 10,000 cells/ml is added aseptically to each flask. The volume of inoculum added should be between 0.1 and 1.0 ml.

2. Incubation. Incubation conditions for the test alga are given in Table 4.7. Test flasks are incubated for 120 hours.

TABLE 4.7 INCUBATION CONDITIONS FOR FRESHWATER ALGAL ASSAY ORGANISMS

Species	Light Intensity, lumens/m^2	Photoperiod Hours of Light:Dark	Shaking Speed, Oscillations Per Minute	Temperature, °C
Selenastrum capricornutum	4300 ± 650	24:0	100	24 ± 2

3. Response monitoring. After 120 hours of exposure, algal growth is measured by either of the following methods: (a) electronic particle counting or (b) direct counting. Cursory microscopic observation is desirable to reveal any abnormalities in cell shape or condition.

a) Electronic particle counting. A Model ZBI Coulter electronic particle counter with mean cell volume computer (MCV/MHR) is used. A C-1000 Channelyzer may be used rather than the MCV/MHR, but it is neither preferred nor recommended. The MHR Computer must be calibrated with the Organic Calibration material; biomass may be determined indirectly by the following equation:

$$\text{S. capricornutum mg dry weight } l^{-1} = [(A) - (P)] \times \text{MCV } (\mu m^3) \times F$$

where A is the algal cell counts ml^{-1}, P is the particulate counts of the blank ml^{-1}, MCV is the mean cell volume and F is the correlation coefficient to be determined by each laboratory.

If there are particles in the test material, it is usually possible to eliminate counts contributed by the particles from the total counts. If the particles are in the same size range as the algal cells, the blank flasks are counted and these counts subtracted from the total counts.

The advantage of this method is that it allows for determination of biomass produced in addition to cell numbers.

b) Direct counting. A hemacytometer counting chamber and a microscope are used. Two samples are taken from each flask, and two counts are made of each sample. Whenever feasible, 400 cells per replicate are counted in order to obtain ± 10 percent accuracy at the 95 percent confidence level. This method permits visual inspection of the condition of algal cells and discrimination between algal cells and debris or particulates in the test material.

4.3.5 Results and Data Interpretation

Calculations. Percent inhibition (I), or stimulation (S), is calculated for each concentration according to the following formula:

$$\text{Percent Response} = \frac{T - C}{C - IN} \times 100 \qquad \begin{matrix}\text{Positive response = Stimulation} \\ \text{Negative response = Inhibition}\end{matrix}$$

where C is the mean growth in the control (mg l^{-1}), T is the mean growth in the treated culture (mg l^{-1}) and IN is the dry weight of inoculum used (mg l^{-1}).

Different endpoints may be calculated from the percent response vs. concentration data. For samples which are inhibitory, an an EC_{50} (defined as the lowest test concentration causing growth inhibition of 50 percent relative control) is calculated. For samples which are stimulatory, an SC_{20} (defined as the lowest concentration causing growth stimulation of 20 percent relative to control) is calculated.

A linear regression analysis is performed on the test results. The X-axis should be percent effluent and the y-axis should be percent effect on growth. Results of linear regression analysis are used to calculate the concentration of effluent causing any effect on growth inhibition or stimulation) by the following formula.

$$T = \frac{\pm E - \pm c}{s}$$

T = Concentration of test material.
s = Slope of regression analysis
c = Constant from regression analysis.
E = Endpoint tested (i.e., EC_{50}, SC_{20}).

A predicted NOEC, defined as the highest test concentration in which growth was not significantly different from that in the control, may also be calculated. The methods of Dunnett (36,37) or Williams (38,39) are recommended for determining the NOEC.

The 120-hour EC_{50} results are evaluated according to criteria defined in Table 4.11 which will permit the test material to be ranked by toxicity category.

4.4 STATIC ACUTE TOXICITY TESTS WITH MARINE FISH AND MYSIDS

4.4.1 Introduction and Rationale

The methods recommended for static acute toxicity tests on marine fish and mysids provide a large volume of data in a short period of time. Principles of Level 1 testing with marine organisms are similar to testing with freshwater organisms, as described in Section 4.2.

The recommended test animals in marine tests are the juvenile sheepshead minnow, *Cyprinodon variegatus*, and the adult mysid, *Mysidopsis bahia*. The recommended tests for both species are static acute exposures that allow calculation of the 96-hour LC_{50} for fish and the 96-hour EC_{50} for mysids. Because death is not always easily determined with some invertebrates, an EC_{50} is calculated rather than LC_{50} for the mysid.

The following procedures have been adapted largely from References 21 and 40.

4.4.2 Materials and Methods

General procedures listed for all aquatic tests in Section 4.1.1 are applicable to the static acute toxicity tests with marine fish and mysid. Specific areas discussed in Section 4.1.1 that should receive careful attention are: facilities, construction materials, test containers, cleaning and preparation of glassware, receipt and quarantine for fish, disease treatment for fish, test material and dissolved oxygen concentration. Materials and methods unique to marine fish and mysid tests are as follows.

Dilution water. Artificial sea salts* are used for preparation of marine dilution water. Salts are added to glass-distilled or deionized water to attain the appropriate salinity which is confirmed with a salinometer. Mysid tests are conducted at a salinity of 22-26 parts-per-thousand (ppt) and sheepshead minnow tests are conducted at a salinity of 10 parts-per-thousand.

The dissolved oxygen concentration of dilution water should be between 90 and 100 percent saturation. Water that may be contaminated with undesirable microorganisms should be passed through an ultraviolet sterilizer.

4.4.3. Test Organisms

Species. The species to be used for Level 1 marine tests are the juvenile sheepshead minnow (*Cyprinodon variegatus*) and adult bay mysid (*Mysidopsis bahia*).

Source. Mysids may be collected from wild populations in relatively unpolluted areas, purchased from commercial suppliers, or cultured in the laboratory according to the method of Nimmo *et al*. (41).

Juvenile sheepshead minnows may be cultured according to the method of Schimmel *et al*. (42) or purchased from commercial suppliers.

Culturing, care and handling. Methods for handling, rearing and static testing of the mysid are given in Nimmo *et al*. (41) and Borthwick (43). Schimmel *et al*. (42) describes a method for culturing sheepshead minnows. These references should be consulted prior to establishing laboratory culture systems for both mysid and sheepshead minnows.

During holding, acclimation and testing, the organisms must not be disturbed unnecessarily, either by excessive handling or excessive movement around the tanks. Handling should be done as gently, carefully and quickly as possible.

Stock fish should be held at 20 ± 2°C. If they are collected at another temperature, they should not be subjected to more than 2°C change in temperature in any one-hour period or to more than a 5 ppt change in salinity (the fishes will be raised at 10 ppt) in any 24-hour period. Crowding during acclimation must be avoided. Commercial flake food† should be fed to the fish once a day. The fish should show no signs of stress such as discoloration, altered behavior or disease and must be kept for at least two days in acclimation tanks.

Holding temperature for mysids is between 22 and 25°C with a salinity range of 22 to 26 ppt; they should be cultured and maintained within these limits.

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*For example, Rila Products, Teaneck, NJ 07666

†For example, Longlife Aquarium Products, Harrison, NJ 07029.

Mysids must be fed approximately two live 48-hour-old *Artemia* nauplii per mysid each day. It is imperative to maintain a sufficient quantity of food in the culture system at all times to prevent cannibalism.

A group of fish or mysids must not be used for a test if individuals appear to be diseased or otherwise stressed, or if more than five percent die within the 48 hours immediately prior to the beginning of the test. If a group fails to meet these criteria, all individuals must be discarded or treated and held an additional four days. It may be more practical to discard the entire group.

4.4.4 Experimental Design

Test Procedure. Marine aquatic ecological assays with fish and macroinvertebrates are parallel in design to the corresponding freshwater tests. Ten fish and 10 mysid per replicate are exposed to six concentrations of test material for 96 hours. There should be a minimum of two replicates for fish and three replicates for mysid for each test concentration or control. Use of more test organisms and replicate test containers as well as random assignment of test organisms to containers is desirable.

The highest dose should be at the maximum applicable dose (MAD) for that sample type (see Table 4.11) unless physical characteristics of the sample, sample size or previously gathered toxicity data contravenes this. The concentrations should be in a geometric series, each one at least 50 percent of the next higher.

The sheepshead minnow tests should be conducted at 20 ± 2°C and those with mysid at 22 to 25°C. A photo period of 16 hours light and 8 hours dark is used for the sheepshead minnow and continuous light for the mysid. The fish should not be fed for 48 hours before the beginning of a test, or during the test. However, mysids require food and must be fed during acclimation and testing. They should be given approximately 20 48-hour old *Artemia* nauplii (per 10 mysids) daily. The test conditions are summarized in Table 4.8.

Each test requires a negative control with the same dilution water, conditions, procedures and organisms as used in the test concentrations. If any solvent other than water is present in the test concentration, a solvent control is also required. This solvent control is treated the same as the negative control except that the same amount of solvent used to dose the other test containers is added to the control containers.

In the sheepshead minnow test there should be 15 liters of test solution or control water in each 19.6-liter jar. If 30 x 30 x 60-cm containers are used, the solution should be between 15 and 20 cm deep.

TABLE 4.8 SUMMARY OF TEST CONDITIONS, MARINE FISH OR MACROINVERTEBRATE TEST

	Sheepshead minnow *Cyprinodon variegatus*	*Mysidopsis bahia*
Temperature, °C	20 ± 2	22 - 25
Photoperiod, hours light:dark	16:8	Continuous light
Water quality, salinity, ppt	10	22 - 26
Container size	19.6 liters	2 liters
Test volume	15 liters	1 liter
Organisms per container	10	10
Replicates	2	3
Feed	No	Yes
Duration, hours	96	96
Measurements, hours D.O., pH	0, 24, 48, 72, 96	0, 24, 48, 72, 96
Salinity, hours	0, 96	0, 96

In the mysid test there should be 1 liter of solution or control water in each 2-liter beaker or culture dish.

Organisms should be placed in the test and control vessels not more than 30 minutes after the test solutions are prepared. Chemical, physical and biological data are taken and recorded as described below for the duration of the test.

If no toxicity is detected at any concentration and the MAD dose was tested, then no further testing is required. The test material may be reported as having no detectable toxicity. Test materials that kill or immobilize all or nearly all the test organisms should be retested with a lower dose range.

Biological loading. The biological loading in each test and control vessel should not exceed 0.8 g of test organism per liter or be so high as to (1) reduce dissolved oxygen concentration below 60 percent saturation (2) raise the concentration of metabolic products above acceptable levels or (3) stress the organisms by overcrowding, any of which may invalidate the test results.

4.4.5 Results and Data Interpretation

Chemical and physical data. Dissolved oxygen concentration and pH should be measured at the beginning of the test and every 24 hours thereafter in the controls and in the high, medium and low test concentrations. Temperature of the water bath or controlled-temperature area should be recorded continuously or every 24 hours.

Concentration of un-ionized ammonia, if required, can be obtained by measuring total ammonia and consulting Reference 30.

Methods for the foregoing tests are described in References 21 and 43.

Biological data. Mortality is the effect most often used to define acute toxicity to aquatic organisms. Criteria for death are usually lack of movement, especially of gill movement in fish, and lack of reaction to gentle prodding.

Because death is not always easily determined with some invertebrates, an EC_{50} may be calculated rather than an LC_{50}. The principal criterion for effect on mysids is immobilization, defined as lack of movement except for strongly diminished activity of appendages.

Death or immobilization and abnormal behavior should be recorded every 24 hours for the duration of the test. Dead organisms should be removed as soon as they are observed. If more than 10 percent of test organisms in any control die or are immobilized, the entire test is unacceptable. Observations of test materials, which produce harmful effects *in vivo*, but do not result in deaths, are difficult to quantitate. Such observations provide insight into the sublethal effects of a sample on aquatic organisms and may be used to recommend further investigation of the test material.

Table 4.5 lists definitions of fish behavior terms and a code for recording and reporting.

Laboratory data forms. Forms for recording chemical, physical and biological data in both sheepshead minnow and mysid tests are illustrated in Quality Control documents (9).

Calculations. The concentration of test material lethal to 50 percent of the population (LC_{50}) and 95 percent confidence limits should be determined (when possible) at 24-, 48-, 72-, and 96-hour exposure for fish tests and the EC_{50} and 95 percent confidence limits at the same time points for the mysid tests. Any of several methods which have been reviewed by Stephan (34) including moving average, Spearman-Kärber, Litchfield-Wilcoxin, probit or binomial may be used. The results are evaluated according to criteria defined in Table 4.11 which permits the test material to be ranked by toxicity category.

4.5 MARINE ALGAE 96-HOUR SCREENING TEST

4.5.1 Introduction and Rationale

The fundamental principles of the marine algae screening test are identical to those described for the freshwater algae screening test (Section 4.3.1).

4.5.2 Methods and Methods

General procedures listed for all aquatic test in Sections 4.1.1. are applicable to the static acute toxicity test with marine algae. Specific areas discussed in Section 4.1.1 that should receive careful attention are: facilities, construction materials, test containers, cleaning and preparation of glassware and test material. Materials and methods unique to marine algal tests are as follows:

Equipment. Equipment should include a constant-temperature room or incubator capable of providing temperature control of 20 ± 2°C. A gyrotary shaking apparatus capable of 60 oscillations per minute should be available for test culture flasks. Illumination of 4300 ± 650 lumens/m^2 (400 ft-C) is required for marine algae with a photoperiod of 14 hours light and 10 hours dark. Overhead cool-white fluorescent bulbs should be used. Light intensity is measured adjacent to the flask at liquid level using a light meter capable of being calibrated against National Bureau of Standards lamps.

Culture containers for this and other aquatic tests are discussed in Section 4.1.1. Erlenmeyer culture flasks of Pyrex or Kimax glass are used in either 125-, 250- or 500-ml sizes. The recommended contents-to-volume ratio for hand-shaken flasks is 1 to 5 and should not exceed 50 percent for continuously shaken flasks. Flask closures must permit gas exchange and prevent contamination. Foam plugs* are suggested. Since some brands may be toxic, each laboratory should determine for each type of closures purchased whether or not there are any significant effects on algal growth.

Support equipment includes an autoclave or pressure cooker capable of producing 1.1 kg/cm^2 (15 psi) at 121°C and a dry-heat oven capable of a temperature of 120 ± 1°C. A high-quality microscope or spectrophotometer is needed for biomass counting.

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*For example, Gaymar Industries, Inc., Orchard Park, NY 14127.

Marine Algal Nutrient Medium. Synthetic sea water is prepared by adding approximately 30 g of a commercial salt mix* to 1 liter of deionized water. Salt mix is added with continuous stirring until the salinity is 30 parts-per-thousand when all the salt is dissolved. Salinity may be measured with a refractometer, salinity-conductivity meter or salinometer.

Nutrients are added to the synthetic sea water. For stock culture medium; 30-ml metal mix, 2.0-ml minor salt mix and 1.0 ml vitamin mix are added to 1 liter (final volume) of synthetic sea water. Composition of these mixes is given in Table 4.9. Metal mix used for stock culture medium contains EDTA whereas that used for toxicity tests does not. To prepare medium for toxicity tests and control, 15-ml metal mix (without EDTA), 1.0 ml minor salt mix and 0.5 ml vitamin mix are added to 1 liter (final volume) of synthetic sea water.

Media are sterilized by autoclaving at 1.1 kg/cm^2 (15 psi) at 121°C for 15 minutes. Culture and test media are cooled and held at least 12 hours to allow the pH to stabilize.

TABLE 4.9 COMPOSITION OF MARINE ALGAL ASSAY MEDIUM (MAAM)

Compound	Concentration
Metal mix	
$FeCl_2 \cdot 6H_2O$	0.048 g/l
$MnCl_2 \cdot 4H_2O$	0.144 g/l
$ZnSO_4 \cdot 7H_2O$	0.045 g/l
$CuSO_4 \cdot 5H_2O$	0.157 mg/l
$CoCl_2 \cdot 6H_2O$	0.404 mg/l
H_3BO_3	1.140 g/l
Na_2EDTA[a]	1.000 g/l
Minor salt mix	
K_3PO_5	3.0 g/l
$NaNO_3$	50.0 g/l
$Na_2SiO_3 \cdot 9H_2O$	20.0 g/l
Vitamin mix	
Thiamine hydrochloride	50.00 mg/100 ml
Biotin	0.01 mg/100 ml
B_{12}	0.10 mg/100 ml

[a] EDTA added only to metal mix used for stock culture medium.

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*For example, Rila Products, Teaneck, NJ 07666.

4.5.3 Test Organisms

The recommended test organism for marine algal assays is *Skeletonema costatum* (22). Algal cultures are available from the Culture Collection of Algae, Department of Botany, University of Texas, Austin, TX 78712.

Stock cultures of algae should be maintained at 20 ± 2°C under a 14-hour light, 10-hour dark photoperiod in marine algae culture medium. It is recommended that several cultures be maintained on agar under axenic conditions. Transfers of stock cultures should be made every six to eight days to provide cultures with a sufficient number of cells growing exponentially for test inoculations.

4.5.4 Experimental Design

Preparation of Toxicant. Depending on its nature, the test material is prepared by one of two methods. The first method is for solids or non-aqueous liquids. These materials may be added directly by weight to the algal medium or a concentrated stock solution may be prepared in deionized water (or other solvent); equal-volume aliquots of a small size are added to each treatment. If it is not possible to prepare a homogeneous solution of the test material, it must be added directly into each replicate flask.

The second method of test material preparation is for aqueous effluents and aqueous leachates of solids. Nutrients are added in the same quantities as in the control medium to 1 liter of the effluent. The effluent is used as the 100 percent test material treatment. Additional test concentrations are prepared on a volume-percent basis by mixing appropriate volumes of effluent medium mixture with control medium. Thus, all test treatments and controls will contain the same amount of nutrients unless the effluent contained nutrients.

Test Procedure. Six concentrations of test material should be prepared, as well as negative controls and solvent control if needed, all with three replicates. A fourth replicate for each treatment contains test material and nutrient medium, but is not inoculated with algae. This blank is necessary to correct biomass measurements for particulates which may be present in the test material.

If a solvent is necessary for the preparation of the test material, a solvent control must be included. All test flasks and the solvent control must contain the same amount of solvent. Solvent levels should be as low as possible so as not to interfere with test results. The toxicity of commonly used solvents should be determined in each laboratory using this bioassay to help select suitable solvent levels.

1. *Inoculum*. A six- to eight-day-old stock culture is used as the inoculum source. Population density in the stock culture is determined by direct counting or spectrophotometry. A volume of inoculum calculated to yield an initial concentration of 30,000 cells/ml is aseptically added to each flask. The volume of inoculum added should be between 0.1 and 1.0 ml.

2. Incubation. Incubation conditions for marine algal assays are given in Table 4.10 below.

TABLE 4.10 INCUBATION CONDITIONS FOR MARINE ALGAL ASSAY ORGANISMS

Species	Light Intensity, Lumens/m^2	Photoperiod, Hours of Light:Dark	Shaking Speed, Oscillations Per Min	Temperature, °C
Skeletonema costatum	4300 ± 650	14:10	60	20 ± 2

3. Response monitoring. After 120 hours of exposure, algal growth is measured by one of the following methods: (a) direct counting, or (b) spectrophotometry. Microscopic observation is desirable to reveal any abnormalities in cell shape or condition.

a) Direct counting. A hemacytometer counting chamber and a microscope are used. Two samples are taken from each flask, and two counts are made of each sample. Whenever feasible, 400 cells per replicate are counted in order to obtain ± 10 percent accuracy at the 95-percent confidence level. This method has the advantage of allowing for the discrimination between algal cells and debris or particulates in the test material.

b) Spectrophotometry. A rapid technique for biomass monitoring is absorbance. This is the preferred method for Level 1 estuarine algal studies. A double-beam spectrophotometer with high-quality cuvettes should be used. A sample is withdrawn from each flask and placed in the cuvette. Absorbance is read against the proper blank for each concentration. Because absorbance is a complex function of the volume, shape and pigmentation of the algae, a calibration curve should be constructed to establish the relationship between absorbance and concentration (absorbance is determined on known dilutions of the control culture). The effect of particulates in the test material, if any, on the absorbance readings should also be determined.

4.5.5 Results and Data Interpretation

Calculations - Percent inhibition (I), or stimulation (S), is calculated for each concentration according to the following formula:

$$\text{Percent Response} = \frac{T - C}{C - IN} \times 100 \qquad \begin{array}{l}\text{Positive Response = Stimulation} \\ \text{Negative Response = Inhibition}\end{array}$$

where C is the absorbance or cell numbers in the control (mg l^{-1}) T is the absorbance or cell numbers in the treated culture (mg l^{-1}) and IN is the dry weight of inoculum used (mg l^{-1}). Different endpoints

TABLE 4.11 DEFINITION OF TOXICITY CATEGORIES FOR AQUATIC ECOLOGICAL ASSAYS

Assay	Activity Measured	Sample Type[a]	MAD[b]	Units	Range of LC_{50} or EC_{50} Concentrations			
					High	Moderate	Low	Not Detectable
Freshwater Fish	96-hr LC_{50}[c] (lethality)	S AL	1 100	g/L percent	<0.01 <20	0.01-0.1 20-75	0.1-1 75-100	ND[d] at >1 ND at >100
Freshwater Invertebrate	48-hr EC_{50}[e] (immobilization)	S AL	1 100	g/L percent	<0.01 <20	0.01-0.1 20-75	0.1-1 75-100	ND at >1 ND at >100
Freshwater Algae[f]	120-hr EC_{50} (growth inhibition)	S AL	1 80	g/L percent	<0.01 <20	0.1-0.1 20-75	0.1-1 75-80	ND at >1 ND at >80
Marine Fish	96-hr LC_{50} (lethality)	S AL	1 100	g/L percent	<0.01 <20	0.01-0.1 20-75	0.1-1 75-100	ND at >1 ND at >100
Marine Invertebrate	96-hr EC_{50} (immobilization)	S AL	1 100	g/L percent	<0.01 <20	0.01-0.1 20-75	0.1-1 75-100	ND at >1 ND at >100
Marine Algae	96-hr EC_{50} (growth inhibition)	S AL	1 100	g/L percent	<0.01 <20	0.1-0.1 20-75	0.1-1 75-100	ND at >1 ND at >100

[a] S = solid, AL = aqueous liquid. Nonaqueous liquids are evaluated on an individual basis because of variations in samples such as solubility in water, vehicle, percent organic vehicle and percent solids. The maximum applicable dose and range of doses able to be tested for nonaqueous liquids is dependent upon the solubility of such samples in water. Because of this variability, evaluation criteria have not yet been developed.

[b] MAD = Maximum applicable dose.

[c] LC_{50} = Calculated concentration expected to kill 50 percent of population.

[d] ND = Not detectable.

[e] EC_{50} = Calculated concentration expected to produce effect in 50 percent of population.

[f] The MAD for routine freshwater algae testing of liquids is 80 percent rather than 100 percent as in the other tests. Evaluation of low (L) or not detectable (ND) results for this test should take this into account. Samples can be retested at higher concentrations.

may be calculated from the percent response vs. concentration data. For samples which are inhibitory, an EC_{50} (defined as the calculated test concentration causing growth inhibition of 50 percent relative to control) is calculated. For samples which are stimulatory, an SC_{20} (defined as the calculated concentration causing growth stimulation of 20 percent relative to control) is calculated. For all samples the no observed effect concentration (NOEC) is established or the EC_{50} or SC_{20} are calculated using the method previously noted in Section 4.3.5. The results are evaluated according to criteria defined in Table 4.11 which permits the test material to be ranked by toxicity category.

4.6. BIOACCUMULATION PROCEDURE FOR INDUSTRIAL AND ENERGY SOURCE SAMPLES

4.6.1. Introduction and Rationale

The need for inclusion of a simple screening procedure in Level 1 environmental assessment to evaluate the bioaccumulation of components in complex mixtures that would accumulate in aquatic organisms has been established. Such a procedure will establish the need to perform subchronic or chronic biological tests at Level 2.

A high-performance liquid chromatographic (HPLC) procedure is available that fills the basic requirements of a screening bioaccumulation test. The HPLC procedure is based on known correlations between octanol/ water partition coefficients (expressed as Log P) and bioconcentration (44). This section describes the procedure to be used on complex mixtures from energy and industrial sources.

4.6.2 Materials and Methods

A high-performance liquid chromatograph equipped with a solvent gradient accessory and a 254 nm ultraviolet detector is employed. The column recommended is a Varian* preparative reverse phase Micropack CH column (part number 03-912152-72) which consists of a 30 cm x 8 mm id stainless-steel cylinder filled with 10 micron lichrasorb to which octadecylsilane is permanently bound. Equivalent columns of the same dimensions are acceptable. A reverse phase guard column (Whatman or equivalent) prior to the preparative reverse phase column is recommended.

The Varian preparative column, operated at room temperature will give the proper separation efficiency when an isocratic mobile phase of 85/15 methanol water at a flow rate of 2.0 ml/min is used. The column should be allowed to equilibrate for at least two void volumes (approximately 10 min) before injection of any sample. After each analysis, the column is cleaned by programming the mobile phase to 100 percent methanol. Organic solvents should be distilled in glass or of equivalent quality. Water should be free of organic contaminants.

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*Varian Associates, Inc., Palo Alto, CA 94303.

Calibration. Seven standards (Table 4.12) are used to calibrate the retention and sensitivity characteristics of individual instruments. These seven standards represent a range of retention times and P values within which the majority of bioaccumulation components of environmental mixtures will elute.

TABLE 4.12 PARTITION COEFFICIENTS OF CHEMICALS USED FOR CALIBRATION

Compound	Log P
Acetone	0.55
Benzene	2.13
Bromobenzene	2.99
Biphenyl	3.76
Bibenzyl	4.81
pp-DDE	5.69
2, 4, 5, 2', 5' - Pentachlorobiphenyl[a]	6.11

[a] Other pentachlorobiphenyl isomers with a minimum of 2 and a maximum of 3 chlorine atoms on a ring are acceptable.

Standards are dissolved in a mixture of acetone and cyclohexane (3:1 v/v). A composite standard is prepared by combining quantities of individual standards. The concentration of each individual chemical in solution is adjusted to give a chromatographic peak of 25 to 75 percent full scale at the highest operating sensitivity of the instrument. (For example, attenuation 1 at 10 mv full scale).

The six standards are prepared in one solution and used to calibrate the elution time in units of Log P. Elution times may differ because of varying void-volume characteristics of HPLC instrumentation.

Sensitivity calibration. For the results from different chromatographic systems to be comparable and to insure a minimum detection sensitivity for specific compounds, the calibration mixture is analyzed prior to each day's accumulation analyses. Daily calibration eliminates irregularities caused by small changes in flow or solvent characteristics and acts to monitor column performance during prolonged use as well.

Sensitivity is based on the average quantity of the calibration compounds which causes a 25-percent scale deflection. The sensitivity is calculated from the geometric mean of the instrument response to the chemicals listed in Table 4.12 with the exception of acetone. This value expressed in μg/25-percent full-scale deflection is defined as Instrument Sensitivity (IS).

4.6.3 Experimental Design

Sample preparation and analysis. Samples should be prepared according to the methods for organic analysis described in the Level 1 sampling and chemical analysis manual (1). Since different types of samples are collected in a source assessment, the amount of sample taken for bioaccumulation analysis must be calculated separately for each type. The quantity of sample used for the analysis is based on the IS. The portions of Level 1 sample required for analysis for each of the four typical samples types acquired in Level 1 are shown in Table 4.13.

TABLE 4.13 SAMPLE QUANTITIES FOR BIOACCUMULATION

Sample	Sampling Method	Quantity for HPLC Analysis
Aqueous Liquid	Grab	Extract from IS liters
Bulk Solid	Grab	Extract from IS g
Fluegas Particulate	SASS[a] Cyclone	0.03 x (IS) from SASS $30m^3$ extract
Fluegas Particulate	SASS[a] XAD-2	0.03 x (IS) from SASS $30m^3$ extract

[a] SASS is the source assessment sampling system described in Reference 44.

Sample analysis must be performed under the same conditions as for the calibration mixture. Following elution of the sample components, the column should be flushed with no less than 20 ml of 100 percent methanol. In some cases, additional components are detected during this phase of operation. These components should be recorded as having log P > 6.0. Information acquired from column clean-up must be evaluated carefully since these post run components can arise from the sample, makeup water or methanol eluent, or they may be artifacts caused by a sudden change in eluent.

Following column clean-up, the standard eluent should be passed through the column for a minimum of 10 minutes to allow column equilibration.

4.6.4 Results and Data Interpretation

Log P for each component is determined from the retention calibration curve for that day's analysis. The final output of the analysis is a list of retention times and the corresponding Log P for components which exceed Log P of 3.5 and the 25 percent full scale sensitivity criterion. Components eluting in the column clean-up should be reported as Log P > 6.0.

Interpretation of results from this analysis must be made together with results from Level 1 bioassays, particularly the ecological effects bioassays (Chapters 4 and 5). If the toxicity bioassays show a response, then further effort at Level 2 first should address the cause and control of the toxic response. If the acute toxicity bioassays show no response, but bioaccumulation results are positive (i.e., component(s) with Log P > 3.5), then further effort at Level 2 should deal with the potential chronic effects of the effluent.

Notes on bioaccumulation analysis. Since the capacity and efficiency of the reverse phase column are necessarily high, the analysis demands a precision-packed column. A reverse phase guard column used in series prior to the bioaccumulation column is recommended to extend the useful lifetime of the analytical column.

Under normal operating conditions, the concentrations necessary to meet the 25-percent scale deflection are on the order of those listed in Table 4.14.

TABLE 4.14 APPROXIMATE CONCENTRATIONS FOR CALIBRATION SAMPLES

Compound	Concentration (mg/ml)
Acetone	0.3
Benzene	0.5
Bromobenzene	0.01
Biphenyl	0.2
Bibenzyl	0.2
2, 4, 5, 2', 5' - Pentachlorobiphenyl	0.2

Examples. Examples of quantitites of samples required for this analysis assuming an IS of 1.0 are described here. Aqueous samples are prepared for analysis by extraction at high and low pH with dichloromethane. For sufficient sensitivity, the extract from 1.0 liter of sample is required (i.e., IS liters of sample).

Flue-gas samples are ordinarily taken in quantities sufficient for chemical and bioassay analyses. The standard Level 1 flue gas sample is taken with the SASS train and represents 30 m^3 of flue gas. In order to meet the sensitivity requirements for bioaccumulation (assuming, for example, IS = 1.0), a sample representing 1.0 m^3 of flue gas is analyzed. For 30 m^3, an aliquot of 1.0/30 or .03 of the total SASS sample should be analyzed.

CHAPTER 5

LEVEL 1 TERRESTRIAL ECOLOGICAL TESTS

5.1 INTRODUCTION AND RATIONALE

The Level 1 terrestrial ecological effects tests include assays for determining toxicity of complex wastes in plant and insect test organisms. The tests are able to detect sublethal toxic response to stress in plants (PSE test), sublethal and lethal toxic responses in germinating seeds (RE test) and acute toxicity and reproductive impairment in insects (IT test).

These tests were selected to provide a range of terrestrial organisms for assessing the effect of effluent streams on the environment. Test organisms include maturing plants, germinating seeds and insects. This group of tests offers testing capabilities for all sample types (including gases) with the advantages of low cost, reproducibility and relatively rapid performance time. A future goal for this manual is to include a test procedure for assessing the impact of effluent samples on soil microorganisms (decomposers).

Terrestrial ecological tests are used to determine the concentration of test material that produces a defined toxic effect on a specified percentage of the test organisms in a fixed amount of time. The plant-stress-ethylene test (PSE test) is designed to assess and rank the toxic effects of gaseous effluents on plants by measuring the stress ethylene of plant response and by assessing relative foliar injury in exposed plants. The root-elongation test (RE test) measures the inhibition of root elongation and seed germination. Although both parameters are observable toxic responses and are reported, root-elongation inhibition is the preferred end point. The concentration which inhibits root elongation by 50 percent of the control (EC_{50}) is estimated and used to rank effluent samples. The insect-toxicity assay measures the acute toxicity and reproductive capacity of fruit flies treated with environmental samples. The dose lethal to 50 percent of the flies (LD_{50}) compared to the control is calculated and used to rank test samples. In the optional fertility test, the effective concentration which reduces the fecundity of surviving dosed flies to 50 percent of control flies (EC_{50}) is calculated. Characteristics of the Level 1 Terrestrial bioassays are given in Table 5.1.

The Level 1 terrestrial tests represent the state of the art for environmental assessment for terrestrial ecological effects. These tests have not been as thoroughly validated with complex environmental mixtures as have the health and aquatic ecological effects tests. Because of the lack of published procedures and data, a workbook of detailed protocols for Level 1 terrestrial tests has been published (45). Procedures and evaluation criteria may be modified as experience is gained in the future.

TABLE 5.1 CHARACTERISTICS OF LEVEL 1 TERRESTRIAL ECOLOGICAL EFFECTS BIOASSAYS

Characteristic	Plant Stress Ethylene Test	Root Elongation Test	Insect Toxicity Test
Species	Bush Bean	Cucumber, Wheat, Red Clover, Radish, Lettuce	*Drosophila melanogaster*
End Point(s) Measured	Metabolic stress evidenced by ethylene production	Root length	Lethality, Reproductive capacity
Amenable to Sample Types	Gases, Liquids	Liquids, Solids (leachates)	Liquids, Solids
Data Expression	Positive or Negative	EC_{50}	LD_{50}
Special Features	Only validated Level 1 Bioassay for gases; sensitive	Detects toxicity to terrestrial producers; multiple species	Detects lethality to terrestrial consumer plus can provide data on fertility

5.2 PLANT STRESS ETHYLENE TEST

5.2.1 Introduction and Rationale

This test is designed to assess the toxic effects of gaseous effluents on plants by measuring the plant-stress-ethylene response. Under normal conditions plants release low levels of ethylene which function hormonally in the regulation of growth and development. In response to various stresses, ethylene production increases substantially (46). Critical to the effectiveness and applicability of the PSE test is that induction of stress ethylene is proportional to the intensity and duration of the stress over a wide range of stresses (47). Ethane evolution may also increase in response to some stresses and is thought to indicate more severe damage and tissue autolysis (48,49,50). Current evidence suggests that ethylene is a produce of methionine metabolism and the same biochemical pathways are involved in the production of basal and stress-induced ethylene (51,52). Ethane may be produced by peroxidation of linolenic acid upon extensive wounding; this peroxidation may also result in some ethylene production (52).

5.2.2 Materials and Methods

Gas sampling. Gas samples are collected in ten evacuated 150-liter polyvinyl fluoride film bags (Tedlar* or equivalent) which are cleaned and leak tested before use. A minimum of 1365 liters of gas are required for the PSE test. To facilitate handling and to keep the samples in darkness, the bags are contained within 55-gallon fiber drums. Access to the sample bag is through a gate valve attached to the bag and to the metal drum lid.

Sample bags are filled individually during sampling using an additional gate valve attached to the outside of the drum and to the stack sample port with Teflon tubing. During sampling, pressure within the bag is monitored to follow the filling procedure and to prevent bags from bursting.

The sample drums are promptly shipped to the testing laboratory; samples should be tested within three days. If a longer storage period is necessary, the sample is stored at 4°C and permitted to warm to the testing temperature prior to test initiation.

Exposure chamber. Exposure chambers (Figure 5.1) used for this test are similar to those described by Heck *et. al*. (53). A negative-pressure single-pass flow system is used to draw gas into the chambers which are then closed to allow static exposure of the plants to the test gas. Each of the chambers is attached to the test gas intake and exhaust manifolds (Figure 5.1). The intake manifold terminates a junction permitting attachment of the gas-sample-container manifold to which the 10 sample containers are attached. A high-pressure blower in the exhaust system is used to draw the sample being tested into the chamber. An internal air-circulation system provides air circulation during exposure and prevents concentration gradients from developing within the chamber. Inlet tubing and fittings are made of polypropylene to reduce corrosion and contamination; exhaust fittings are made of polypropylene, Tygon, and polyvinylchloride. The operating characteristics of this system are similar to those reported by Heck *et. al*. (53).

Plant culturing. Bush beans, *Phaseolus vulgaris* L., cultivar Harvester, are grown from seed for use in the PSE test. A total of eight plants are required for each test gas concentration and each control. It is required that 12 be grown for each condition so that uniform plants may be selected. Two seeds are planted in 10-cm plastic pots using promix BX or Jiffy Mix as the potting medium. The seedlings are thinned to one per pot seven to eight days after seeding. Plants are surface-watered daily with sufficient half-strength Hoagland's nutrient solution (54) to require the excess solution to drain. They are maintained at average day/night temperatures of 26°C and 20°C, respectively. A 16-hour photoperiod is recommended but a minimum 12-hour photoperiod is acceptable. During the photoperiod, the light intensity must be a minimum of 100 g cal/cm^2/day (200-2500 nm) as measured at plant height. An average light intensity

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*E.I. Dupont de Nemours and Co. (Inc.), Wilmington, DE 19898.

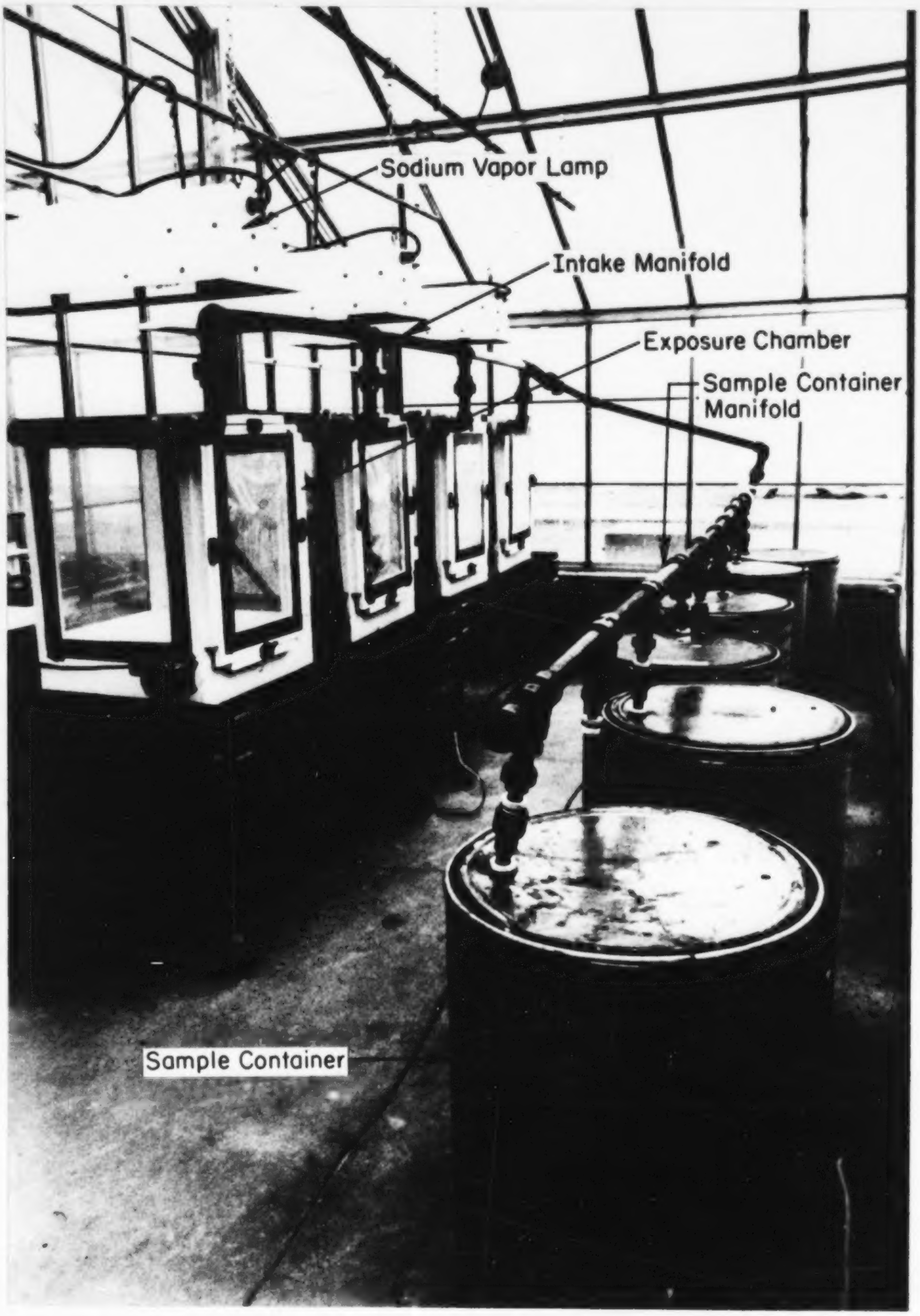
Sodium Vapor Lamp
Intake Manifold
Exposure Chamber
Sample Container Manifold
Sample Container

Figure 5.1 COMPLETE STRESS ETHYLENE EXPOSURE SET-UP SHOWING THE VARIOUS COMPONENTS AND THEIR INTER CONNECTIONS (only 6 of the 10 sample containers are shown here)

of 200 g cal/cm^2/day is preferred. When bush beans are grown in a controlled-environment chamber, air is continuously renewed to prevent CO_2 depletion. The plastochron index (55,56) is used to select plants for assay to ensure that they are exposed at a standard time of development and to ensure population uniformity. Bush beans are exposed within 24 hours of the time when the population to be treated exhibits a plastochron index of 1.5 ± 0.3.

5.2.3 Experimental Design

Exposure. Bush beans are exposed to three concentrations of test gas, with charcoal-filtered air used as a negative control and chlorine gas used as a positive control. The exposure is carried out sequentially at room temperature with the control chamber being run first, followed by the experimental chambers and then positive controls. Exposures to all test gas concentrations are begun within one hour of the start of controls. The objective is for each chamber to contain twice as much test gas as the chamber with the next lower concentration. The mixing curve for the test gas in the chamber air is logarithamic. Thus, to double the concentration x which results from a mixing time of y sould require doubling the mixing time (2y). To double the concentration again (4x) would require eight times the mixing time (8y). The actual mixing time for any set of chambers will be a function of the rate of flow of gas through the chamber and the total quantity of test gas available. The mixing curve for any chamber design should be empirically determined using an easily detectable reference gas. Methane at 80 parts-per-million may be used and the change in chamber concentration measured with a hydrocarbon analyzer.

The flow rate for each chamber is calibrated prior to exposure. A rotameter is placed in the exhaust system between the blower and the chamber and used to calibrate the flowmeter of each chamber. A flow of 5 cfm (142 l/min) is established using the rotameter for reference; the reading in centimeters of water on the manometer connected across the orifice is recorded. The rotameter is subsequently removed and the flow adjusted with the regulating valve to achieve the previous reading on the manometer, completing the calibration.

The exposure chambers are prepared by placing eight plants in each chamber (being sure that adjacent plants do not touch or shade one another) and activating the internal blowers. The negative control chamber is filled first. The external blower is turned on, the valves on the control chamber are opened, and charcoal filtered room air is drawn through the chamber from the open intake manifold for two minutes.

Filling of the experimental chambers is conducted analogously. The sample-container manifold is connected to the ten gas-sample containers and to the intake manifold. The valves on the first experimental chamber are opened and the test gas is drawn through the chamber for seven minutes. The remaining two experimental groups are exposed in the same fashion, except that the test gas is drawn in for two minutes and one minute, respectively. The concentration of gas doubles from one minute to two minutes of mixing and again from two to seven minutes. Positive-control chambers are filled with filtered room air as are the negative control chambers. Sufficient chlorine gas, calculated to give a chamber concentration of 15 parts-per-million, is injected directly into the chamber via a syringe. Once filled, each chamber is then closed and permitted to incubate for two hours under a 400-W high-pressure sodium-vapor light at a temperature of between 22 and 28°C. After two hours of exposure to the test gas, or control gas mixtures, the contents of each chamber are exhausted through a charcoal filter and the chambers are opened.

Incubation. Immediately after exposure, plants are carefully removed from the chambers and are visually inspected for signs of damage. The plants are then individually sealed with B-620 bags* (Cryovac; 10 x 32 in.; std. gauge) with a wire support surrounding the plant to prevent the leaves from touching the bag (Figure 5.2). The bags should be sealed at a standard position so as to provide a constant volume for ethylene accumulation. Plants are bagged on a laboratory cart so that further handling of the enclosed plants is minimized. The enclosed plants are incubated at 26°C in the dark for four hours while ethylene accumulates within the bag.

Ethylene and ethane analysis. Ethylene and ethane are quantified by gas chromatography using a flame ionization detector (57). A Porapak-N column (2.4 m x 3.2 mm, stainless steel, 80/100 mesh) is used for the separation and is operated isothermally at 80°C. The injection port and detector are maintained at 200°C and helium (40 ml/min) is used as carrier gas. Maximum sensitivity of the instrument is required, as the limit of detection should be 5 parts-per-billion. The gas chromatograph is calibrated on each day the samples are to be analyzed using a minimum of five ethylene standards ranging from 10 to 1000 parts-per-billion to ensure linearity of response. (The response for ethane is assumed to be equivalent to the detector response for ethylene.) Peaks are identified by retention time compared to standards and may be documented further by cochromatography with standard ethylene and ethane. A 1-ml sample is withdrawn from the bag surrounding the plant using a 1-ml gastight syringe and is analyzed by gas chromatography. Ethylene concentrations for negative-control plants exceeding 50 parts-per-billion indicate improper plant handling and the test is repeated. For best results, the standard deviation of the mean ethylene production for each control and experimental exposures should not exceed 1.6.

Assessment of plant injury. At the conclusion of ethylene/ethane analysis, plants are removed from the bags and are maintained for three days using the growth conditions previously specified. Plants are then reinspected for injury. Relative foliar injury as compared to negative controls is expressed on a scale of 0 to 10 with each increasing unit being equal to 10-percent injury of the total leaf area. The types of injury (e.g., leaf lesions, yellowing) should also be noted. In the assessment of plant injury, any slight foliar injury must be reported as 1 (10 percent). Further, only the affected area is to be quantitated. For example, although an entire leaf may be spotted, the injury is assessed as 2 if only 20 percent of the leaf surface is necrotic or yellowed.

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*Cryovac Division, W.R. Grace and Co., Box 338, Simpsonville, SC 29681.

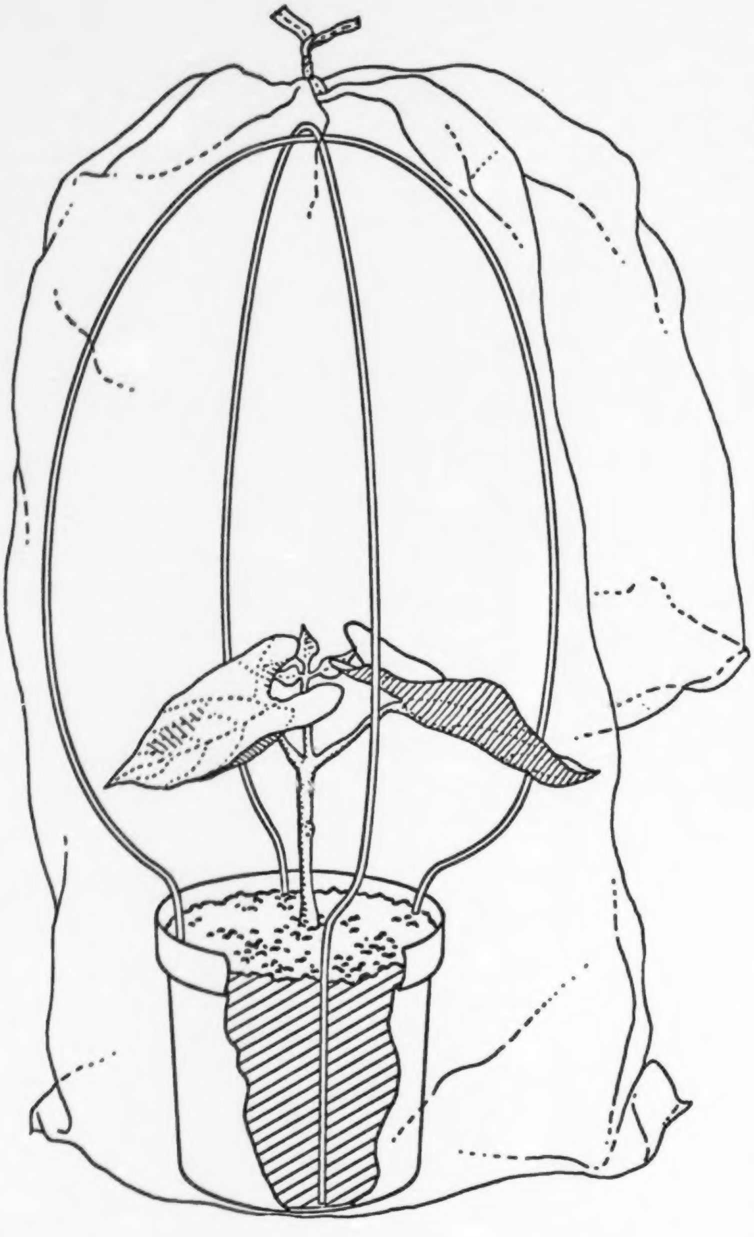

Figure 5.2 Plant Enclosure

5.2.4 Results and Data Interpretation

Ethylene concentrations for plants of a given experimental condition are tabulated and the geometric mean and standard geometric deviation are determined. A stress-response threshold value is determined which is 1.5 times the mean ethylene concentration of the negative-control plants (to account for variability) or 50 parts-per-billion, whichever is greater. A positive response has occurred if the mean for any of the experimental groups exceeds this threshold. The response evaluated by the criteria in Table 5.2 is classed as "high" if the threshold is exceeded by the lowest test gas concentration (one minute); and "moderate" or "low" if the threshold is exceeded in the two-minute or seven-minute mixing times, respectively. Ethane data is monitored and recorded for future reference. Relative plant injury is reported as mean and standard deviation for a given experimental condition noting the type of injury which was observed.

TABLE 5.2 PLANT STRESS ETHYLENE TEST EVALUATION CRITERIA

Toxicity	Lowest Positive Response (Mixing Time in Minutes)[a]	Relative Foliar Injury[b]
High	1	7-10
Moderate	2	4-6
Low	7	1-3
Not Detectable	No response at 7 minutes	0

[a] Dose levels based upon mixing time in minutes are applicable only if flow rates and chamber design given in this chapter are used.

[b] Evaluation based upon foliar injury are tentative and still under evaluation. Each unit is equal to 10-percent injury of total leaf area.

Due to the subjective nature of observation and assessment of plant injury, ranking of test samples based upon relative foliar injury is difficult and should not be used for definitive ranking of test material toxicity. Such information is used by the investigator in the overall evaluation of a sample or for recommendation for additional Level 1 or 2 testing. However, in the case of a highly toxic test gas which may damage the ethylene production mechanism of the plant, foliar damage results are used to corroborate the highly toxic nature of the sample: little or no ethylene production with significant foliar damage (50 percent or greater at the one- or two-minute exposures) confirms a "high" toxicity evaluation. These observations are analagous to the physiological observations of mice in acute *in vivo* rodent tests and to behavior observations of fish in freshwater and marine ecological bioassays.

5.3 ROOT ELONGATION TEST

5.3.1 Introduction and Rationale

The development of seed into a mature plant is a series of complex processes. Assessment of toxic effects requires selection of a stage in plant development that is sensitive to a broad range of toxicants and is important physiologically. Seed germination and root elongation are critical links in plant development beginning with the dormant embryo stage and the period of rapid growth when essential plant structures are formed.

Toxic substances that prevent or reduce germination or root elongation will decrease plant populations and can reduce crop yields. In natural systems, those species affected are less able to compete with other species; thus tolerant species may be selected, resulting in changes in species diversity, numbers and population dynamics.

Inhibition of seed germination and root elongation has been used in determining selective toxicities of herbicides (58,59), screening plants for heavy metal (60,61) and salinity tolerance (62,63) and evaluating toxic chemicals (64,65) and allelopathic substances (66,67). The root elongation/seed germination bioassay has several advantages. It is a rapid test; germination and root elongation can be observed after 115 hours of incubation. It is a simple test that does not require significant investments in equipment and facilities or complicated techniques. Personnel required for performing the bioassay need not be highly skilled.

The same chemical may cause responses at different doses in different plant species (65). To detect an effect from chemicals of unknown toxicity, several plant species should be selected. The species used in this test--lettuce (butter crunch), *Lactuca sativa* L.; radish (cherry belle), *Raphanus sativus* L.; wheat (Stephens), *Triticum aestivum* L.; cucumber (hybrid Spartan valor), *Cucumis sativus* L.; and red clover (Kenland), *Trifolium pratense* L.--are representative of economically important plants of different plant families. Seed chosen germinates, grows rapidly, contains no natural inhibitors and requires no special pretreatment. All test organisms are grown under identical environmental conditions (constant temperature, 25°C, constant dark and enclosed to maintain uniformly high relative humidity).

Although inhibition of root elongation and germination are observable toxic responses, in this bioassay, root elongation inhibition is the preferred end point. Usually, elongation is inhibited at lower concentrations of toxic substances than is seed germination.

5.3.2 Materials and Methods

Facilities. Facilities must include work areas for planting seed and for measurements, preferably isolated from other activities. There should be a fume hood, distilled water source and refrigeration available at 5°C. The test facility must have a seed germinator and a plant growth chamber or some type of controlled environment chamber capable of maintaining a uniform temperature at 25°C within ± 1.0°C.

Test containers. One-piece, molded-glass tanks*, with a 6-quart capacity (approximately 9-½ in. (L) x 6-¼ in. (W) x 7 in. (H)) are used for dosing seeds. Glass plates (5-1/8 in. x 6 in.) of single-strength window glass are prepared with polished edges. The glass plates are supported at a 67° angle in the tank with either a glass rack or glass pegs. The use of glass racks has been found to be superior to the use of glass pegs. The glass rack is constructed from two glass rods (approximately 9 in. long) and six half-circles (4-3/4 in. O.D) of glass tubing connected to the rods at right angles at 1-3/8-in. intervals. The pegs are 2 to 3 cm long and 5 mm in diameter. Twenty pegs are cemented with epoxy to the inside of each glass tank (Figure 5.3).

Equipment. Items specifically needed include a spray bottle with a fog or mist nozzle, metric ruler, forceps, Soxhlet extraction apparatus, triple beam balance, pH meter, osmometer, storage bottles and plastic bags (minimum of 12 in. x 8 in. x 14 in.). An illuminated magnifier may be helpful for planting, seedling examination and root measurement.

Test organisms. The seeds used in the test are available from commercial seed companies, state agricultural experiment stations, and laboratories of the U.S. Department of Agriculture. Seed from one seed lot for each species should be purchased in amounts adequate for one-year's testing. Information on seed lot, seed year or growing season collected and germination percentage should be provided by the source of seed. Only untreated (not treated with fungicides, repellants) seed is acceptable for Level 1 biological testing.

Size grading of seed. After purchase, size-grading is carried out on the entire seed lot for each kind of seed. Small samples of 100-150 g are sized at a time. The seed lot is inspected; trash, empty hulls and damaged seed are removed. Depending on species, a series of four screens is selected to separate samples into size classes (see Table 5.3). The four screens are nested with the screen containing the largest holes on top and screens with successively smaller holes in sequence below. A blank or bottom pan collects the fraction that passes through all screens. Seed is poured onto the top screen and the whole set of nested screens are shaken (by hand or with a vibrator) until all the seed remains on one screen or reaches the bottom pan. The separated fractions are collected and the procedure repeated until all the seed in the lot is sized. That size class which contains the most seed is selected and used exclusively for duration of the tests. The fractions are divided into small lots, placed in separate envelopes or sacks and stored in moisture-proof sealed containers in a refrigerator at 5°C.

Preparation of glassware. The glass tanks (fitted with glass pegs or tanks with glass racks) and glass plates are rinsed in acetone and then thoroughly washed in warm water with a synthetic detergent (e.g. Alconox®).

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*For example, Anchor-Hocking Glass Co., Lancaster, OH 43130.

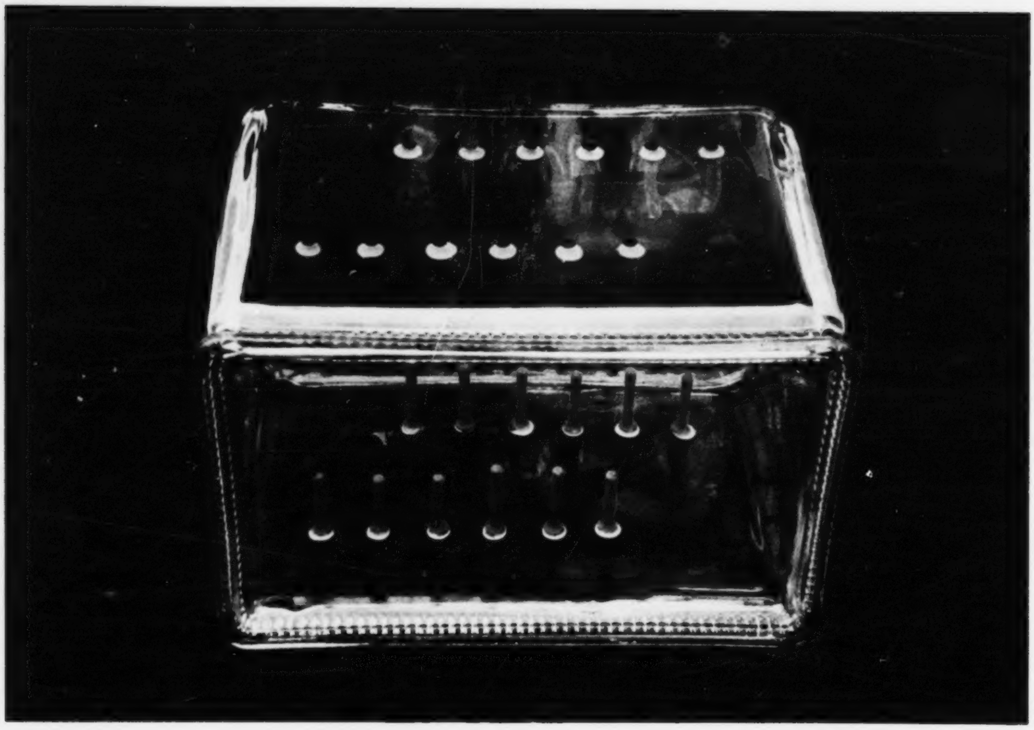

Figure 5.3 Glass Tank With Glass Pegs Cemented in Place

Following washing, the glassware is rinsed in tap water and in 50 percent nitric acid. All glassware is rinsed again thoroughly with tap water and distilled water.

Tissue paper precleaning. Eight to ten sheets of single-ply tissue (Kimwipes®) are placed in a Soxhlet extractor and extracted with distilled water for a minimum of 24 hours. After extraction, the tissues are removed, air dried and stored in a dry container or plastic bag.

TABLE 5.3 HAND SCREENS FOR SIZING SEEDS[a]

Species	Perforated Metal Sheet		Wire mesh
	Round Holes	Oblong Holes	
Red Clover	1/19, 1/18, 1/17, 1/16 (Fractions of an inch)		
Radish	6-1/2, 7, 7-1/2, 8 (64ths of an inch)		
Wheat	9, 9-1/2, 10, 10-1/2 (64ths of an inch)		
Cucumber		1/13 x 1/2 1/14 x 1/2 1/15 x 1/2 1/16 x 1/2 (fractions of an inch)	
Lettuce			6 x 28 6 x 30 6 x 32 6 x 34 (fractions of an inch; e.g., 1/6" x 1/28")

[a] Supplied by (for example), A.T. Ferrell and Co., Saginaw, MI 48601, or Seedburo Equipment Co., Chicago, IL 60607.

5.3.3 Experimental Design

Test Medium. The test medium is an effluent sample or aqueous leachate of a particulate or solid sample. The effluent should be tested as soon as possible after receipt to minimize changing or altering the sample. If it is not possible to test immediately, the sample should be stored at 0 to 4°C in a closed container. Aqueous leachates of solids are prepared using the procedure in Section 2.3.9. Aqueous leachates of solid samples should be tested as soon as possible or the solid sample must be stored in closed polyethylene containers until extraction can be made. Dilutions of the effluent or aqueous extractions should be made without use of solvents or additives except for distilled water, which is used as a negative control. Before testing, the pH of the samples and controls is adjusted to pH 6.5. The highest concentration that can be used is one for which the pH can be adjusted to pH 6.5 using less than 5 ml of 0.1 M KOH or 0.1 M HCl per liter of solution. Test medium osmotic potential should be greater than -3 bars to avoid osmotic effects which can retard root elongation and seed germination. Osmolarity cannot be adjusted except by dilution with water. No more than one percent organic solvent (usually dimethylsulfoxide) should be present in the test sample.

Negative controls should be run using distilled water as the test medium. For a positive control, it is recommended that cadmium chloride be used at a concentration of 15 mg/l for all seeds except wheat, for which 75 mg/l should be used.

Extended Dose Range Test. The extended range-finding test consists of two control tanks, two tanks of 100-percent effluent, and one tank each of 50, 25, 10, 1, 0.1 and 0.01 percent effluent.

A species need not be tested further if both tanks containing 100 percent effluent had mean root lengths of at least 65 percent of control and at least 10 of 15 seeds in one control, 8 of 15 seeds in the second control and 8 of 15 seeds in both 100-percent effluent tanks germinated. Also, in this situation it is not necessary to examine the plates containing this species in the 10- to 0.01-percent tanks. If one or more of the species show mean root lengths less than 50 percent of the control at even the most dilute concentrations, it may be desirable to repeat the test.

Procedure for planting seed. Whatman 3MM chromatography-filter-paper rectangles (5-1/8 x 6 in.) are soaked in the test solution in a shallow tray for a minimum of 5 minutes to saturate. One sheet of filter paper is removed from the test solution, allowed to drain, and placed on a glass plate to which the paper adheres. Trapping air bubbles between the filter paper and the glass plate should be avoided. Using forceps, 15 seeds from one species are placed on the filter paper substrate in a row, equally spaced, across the top of the plate, 1 in. down from the top edge. Seeds are placed with the radicle end toward the bottom of the plate and, in the case of wheat, with embryo side of the seed up. A narrow strip (1-2 cm wide) of previously cleaned single-ply tissue is placed over the row of seeds to hold them in place and, if necessary, sprayed with just enough fine, distilled-water mist to cause the tissue to cling to the seeds and filter paper. Test solution, usually 500 ml, is poured into the rectangular glass tank fitted with glass peg guides (empty tank if glass rack is used). The glass plate holding seed and substrate is inserted in the glass tank between the glass peg guides or in the glass rack to support the plates at a 67° angle (Figure 5.4). The lower end of the plate opposite the seeds should be immersed in the test solution with a minimum of 2 cm, but not more than 3 cm, of the plate and filter paper in the solution. Normally, volumes less than

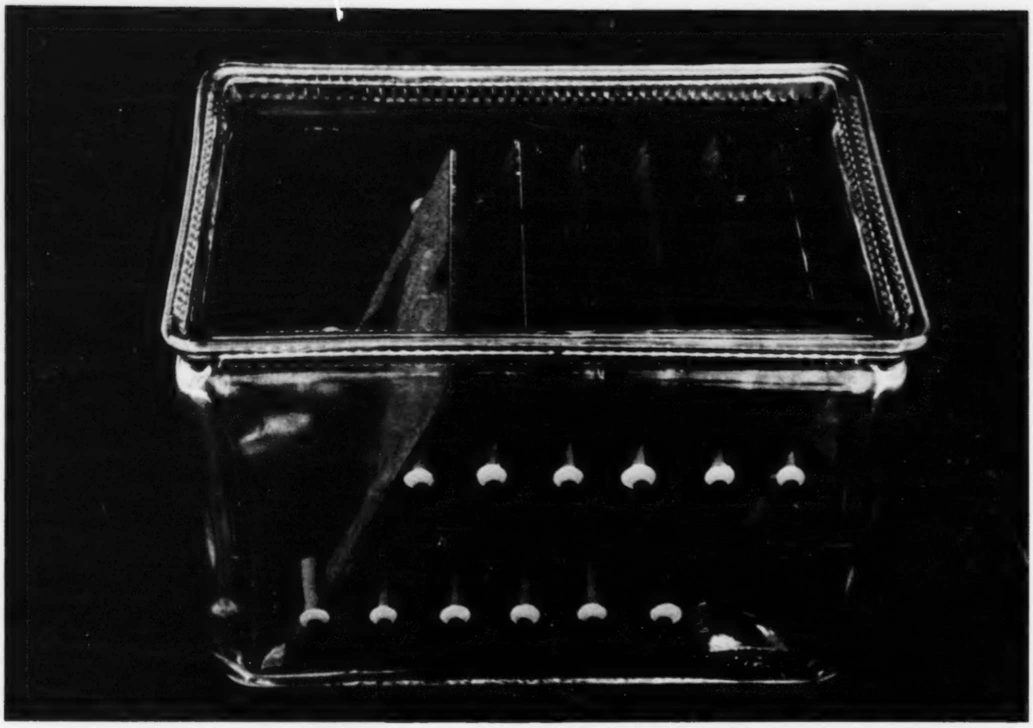

Figure 5.4 **Glass Tank With Glass Plates in Position Between Pegs**

500 ml are not tested, however, samples with less than 500 ml can be tested if needed if clean inert glass beads or marbles are added to the solution to displace and raise the liquid level. This procedure, with one plate of each seed type (cucumber, lettuce, radish, red clover, wheat) per container, is repeated with each test concentration and control.

Incubation. The glass tank, containing five plates with 15 seeds of each species per plate and the test solution, is enclosed in a heavy plastic bag and tied shut. The enclosed tank is placed in the dark, 25°C controlled chamber. A tank is prepared for each test solution of sample effluent and for the distilled water controls and positive controls.

Measurement of root length. Measurement of root length is made at 115 hours from the start of dark incubation. It is important to measure each plate as nearly as possible to 115 hours (not to exceed ± 30 minutes). To measure root length, a plate is removed from the tank and placed on a flat surface. The lengths of all roots are measured to the nearest millimeter and entered on the data sheet. Measurement is from the transition point between hypocotyl and root to the tip of the root (Figure 5.5). At the transition between the hypocotyl and the primary root, the axis may be slightly swollen, contain a slight crook or change noticeably in size (radish, lettuce, cucumber, red clover). In wheat, the single longest primary or seminal root is measured from the point of attachment to the root tip. Additional descriptions and photographs, helpful in making root measurements, are presented in References 45, 68 and 69.

5.3.4 Results and Data Interpretation

Assay acceptance criteria. To estimate accurately the EC_{50} (the concentration which reduces root elongation by 50 percent), the following criteria must be met for each of the species except for any species which showed no effect with 100 percent effluent.

Criteria

1. At least 10 of 15 seeds on one negative control plate and 8 of 15 seeds on the other negative control plate must germinate.

And

2. Each effluent concentration in a series must be at least 50 percent as strong as the next concentration, except for controls.

And At Least One Of The Following

3. There must be at least one effluent concentration for which mean root length was above 65 percent of the control and one concentration for which it was below 35 percent of the control; both of these effluent concentrations must have eight or more seeds germinate. In addition, any concentration more dilute than "above 65 percent" concentration must have a mean root

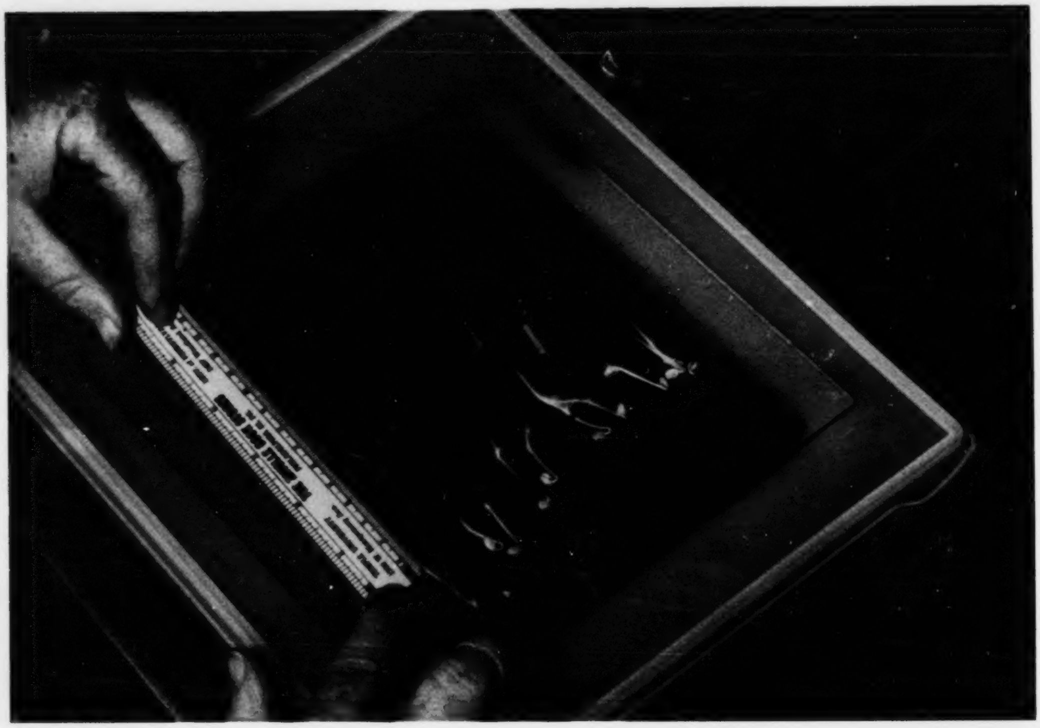

Figure 5.5 Seedlings Showing Method of Measuring Roots

length above 50 percent of the control and any concentration less dilute than the "below 35 percent" concentration must have a mean root length below 50 percent of control.

Or

4. All conditions required in criterion 3 (preceding) must be satisfied with the sole exception that eight or more seeds need not germinate at the "below 35 percent" concentration. However, there must also be one concentration stronger than the "below 35 percent" concentration for which fewer than 8 of 15 seeds germinated.

Or

5. There must be two concentrations each of which have mean root lengths at least 65 percent of the control-at the lower concentration ten or more seeds must have germinated and at the higher concentration five or fewer seeds must have germinated. In addition, there must be one concentration higher than the "five or fewer" concentration for which seven or fewer seeds germinate.

Since most effluents affect root elongation at lower concentrations than germination, criterion 3 will usually be used to satisfy the requirements of the test in addition to criteria 1 and 2. However, in cases where germination is inhibited at lower concentrations than elongation, it may be necessary to use criterion 4 or 5 in place of 3. If a species fails to satisfy criteria 1, 2 and one of 3, 4 or 5, the extended dose range test must be repeated for that species.

Calculation of an EC50 for Root Elongaton by Graphical Interpolation. Provided criteria 1, 2 and 3 are met in the definitive test, the EC_{50} can be estimated in the following manner. For each species which satisfied these three criteria, plot on semi-log paper effluent concentration on the logarithmic axis and percent control mean root length on the arithmetic axis. Draw a straight line between the two effluent concentrations used to satisfy criterion 3. Mean root length will be above 65 percent of control for one of these concentrations and will be below 35 percent of control for the other. The concentration at which this line crosses the 50-percent point for root length is the EC_{50} for root elongation. If no effects were seen with the 100 percent effluent, or if criterion 3 could not be met due to germination inhibition (criteria 4 or 5 instead), it is not possible to estimate an EC_{50} for root elongation.

Reporting. For each of the species either the concentration in (a) or (b) or the quantities in (c) must be calculated and reported.

(a) If the species satisfied criteria 1, 2 and 3, report an estimated EC_{50} for root elongation. Use graphical interpolation to estimate the EC_{50} and rank the test sample using evaluation criteria in Table 5.4.

TABLE 5.4 ROOT ELONGATION TEST EVALUATION CRITERIA[a]

Toxicity	Aqueous Liquids (EC_{50} in Percent Effluent)
High	<0.01
Moderate	0.01-1
Low	1-100
Not Detectable	>100

[a] Criteria for aqueous liquids are tentative and still under evaluation. Criteria for other sample forms (such as soluble solids and aqueous leachates of insoluble solids) are under development.

(b) If the species satisfied criteria 1 and 2 but not criterion 3 (criterion 4 or 5 used instead), report the lowest concentration for which fewer than 8 of 15 seeds germinated. The EC_{50} cannot be estimated for root elongation or inhibition of seed germination from data in this category. Currently, test samples are not ranked from data of this type.

(c) If the range-finding test showed 100-percent effluent had no effect on a species, report the number of seeds which germinated and percent control mean root length for the two 100-percent effluent tanks. The test sample is ranked as having nondetectable (ND) toxicity.

5.4 INSECT TOXICITY ASSAY

5.4.1. Introduction and Rationale

Drosophila melanogaster is a common insect species in nature; this organism possesses many features which make it attractive as an in vivo test system for detecting environmental toxicants. Among these features are: 1) a short life-cycle time of 12-14 days; 2) minimal space, monetary and manpower requirements to maintain stocks; 3) ability to detect toxic effects at specific life-cycle stages (adult, germinal and developmental stages); 4) a well-defined genetic system which makes the detection of specific genetic end points possible, and 5) ability to biotransform genotoxic chemicals via in vivo metabolic enzyme systems. Treatment methods which are normally encountered in animal studies (such as feeding, aerosol inhalation and test-article injection) can also be applied to Drosophila, although feeding is the most common route of exposure.

Drosophila is included in EPA Level 1 environmental assessment testing because its application as a toxicity screening test can be expanded into testing for additional end points. For Level 1 applications, Drosophila will be used to assess acute toxicity in the adults and reproductive capacity among surviving flies after exposure to or treatment with environmental samples. The acute toxicity data will be used for the ranking of effluent streams and identifying sources for further testing. The fertility response will be recorded and may be used to provide possible direction for testing at Level 2.

5.4.2 Materials and Methods

Stocks. Drosophila melanogaster can be easily found in nature but pedigreed stocks may be obtained from the Drosophila Stock Center*. One-day-old wild-type Oregon R (Ore R) male flies are used for exposure to or treatment with environmental samples. One-day-old Ore R virgin female flies are used for mating and egg production in fertility tests. Instant Drosophila medium† (Formula 4-24 without dyes) is used in preparing culture bottles and vials. The flies are maintained on the instant medium in 8-dram glass vials plugged with nonabsorbant cotton. Stock cultures should be placed on new medium every two or three weeks. The flies can be immobilized with CO_2 or ether. The biology and handling of Drosophila melanogaster are described in References 70 and 71.

5.4.3 Experimental Design

Feeding study. Drosophila may ingest particles suspended in liquid but generally are exposed to liquid or solid samples in solution. Liquid environmental samples are tested with various solvents such as water, dimethylsulfoxide (DMSO) and ethanol to determine the most suitable solvent or feeding medium. Water-soluble samples are dissolved directly in a one-percent-sucrose feeding solution. Liquid samples that are not soluble in water are solubilized in ethanol or DMSO and then added to a sucrose feeding solution. The feeding solution is applied evenly to filter paper at appropriate concentrations. The filter papers are allowed to dry and are then placed in a dosing vial as shown in Figure 5.6. Liquid samples not soluble in water, ethanol or DMSO are mixed into a thick paste made with yeast extract and one percent-sucrose feeding solution to form an emulsion mixture which is fed to the flies by applying it to the filter paper liner.

Solid samples are dissolved in one percent-sucrose feeding solution if soluble. Otherwise they are processed into fine particles no larger than 5 µm (Section 2.3.1) and mixed with one percent-sucrose solution to form a suspension. Both solutions and suspensions are applied to filter-paper liners for feeding.

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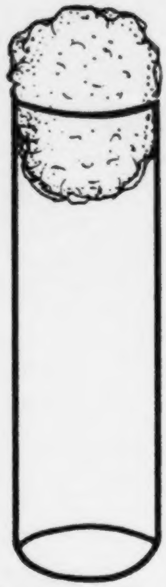
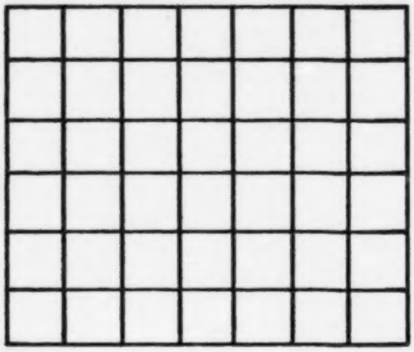
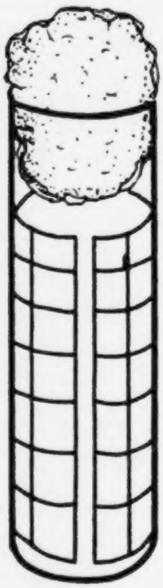
*Drosophila Stock Center, Bowling Green, KY 42101.
†Carolina Biological Supply Company, Burlington, NC 27215.



Glass Culture Vial
Plugged With Cotton Plug



Filter Paper Liner[Solubilized compound is added , spread evenly, and dried.]



Vial Prepared For Exposure. [Fifty flies are added to the vial for 24 hours.]

Figure 5.6 PREPARATION OF TREATMENT CHAMBERS FOR DROSOPHILA

Flies are maintained in dosing vials for 24 hours. The number of flies surviving treatment at the end of the exposure period are counted and living flies are transferred to new vials with medium to measure reproductive capacity (Fertility Test).

Range finding. For feeding experiments, groups of 50 male flies which have been starved for two hours, are placed in vials containing 1.5 ml of feeding solution on filter paper at test-agent concentration levels of 0, 0.1, 1, 10 and 50 mg/ml for solids and 0, 1, 10, 100 and 500 µl/ml for liquids, or containing 5 g of yeast paste with test agent concentration levels of 0, 0.1, 1, 10 and 50 mg/g or 0, 1, 10, 100 and 500 µl/g. The vials are scored for dead flies immediately after the 24-hour exposure period and a range of concentrations for LD_{50} evaluation is developed including levels of the test sample which produce some lethality.

Definitive test for LD_{50} determination. For feeding studies, the LD_{50} determination is performed by using five equally spaced concentration levels derived from the information obtained from the range-finding test. A total of 150 starved male flies, 50 per culture vial, are fed the test sample at each specified concentration level for 24 hours.

Dead flies are counted immediately at the end of the feeding or exposure period and the 24-hour LD_{50} and its upper and lower limits are calculated according to Litchfield and Wilcoxin (20).

Optional fertility test. Twenty-five to fifty male flies obtained from each dose level of the toxicity tests are individually mated to three virgin females in food vials. After four days, the adults are discarded. Vials are examined daily for five to eight days after the exposure for evidence of larval activity. Absence of larvae or lack of normal larval activity as compared with control flies will indicate a decrease in fertility of the sample-treated group. In situations where the difference in larval activity is only marginal, the number of emerged flies per vial will be counted after another seven days. Fertilities of various dose levels are reported for each sample. A more detailed study using a brood-pattern analysis to determine germ-cell stage specificity may be used as follow-up study if the sample shows significant reduction in fertility. Fertility studies provide information on the toxic effects of chemicals on the egg or sperm cells of exposed adults and may be a more sensitive indicator of toxicity than lethality in the adult. The effects of chemicals on fertility can occur at any of several distinct phases including hatching of eggs, larval activity, or metamorphosis.

5.4.4 Results and Data Interpretation

Using the maximum applicable dose (MAD) concept developed for Level 1 screening bioassays, the response of chemicals in the *Drosophila melanogaster* test may be ranked as high, moderate, low or not detectable. Table 5.5 gives MAD and response ranges for the *Drosophila* Level 1 Insect-Toxicity Assay of environmental samples.

TABLE 5.5 DROSOPHILA INSECT TOXICITY ASSAY EVALUATION CRITERIA[a]

Activity Measured	MAD[a]	Response Ranges[b]			
		High	Medium	Low	Not Detectable
Lethality (LD_{50})					
Solid Samples	50	<0.5	0.5-5	5-50	>50
Liquid Samples	500	<5	5-50	50-500	>500
Fertility (EC_{50})					
Solid Samples	50	<0.5	0.5-5	5-50	>50
Liquid Samples	500	<5	5-50	50-500	>500

[a] Concentrations are in mg/ml for solids and slurries and μl/ml for liquid samples tested as solutions and suspensions. Insoluble liquids are reported as mg or μl per gram of yeast extract paste.

[b] Assay evaluation criteria are tentative and under evaluation.

CHAPTER 6

LEVEL 1 DATA FORMATTING AND ANALYSIS

6.1 INTRODUCTION

Data formatting as described here is a guide to organizing data from all Level 1 bioassays into a uniform evaluation format to aid in the use and interpretation of the data. This format is structured so that data can be converted from the conventional bioassay output into four levels of response: Nondetectable (ND), Low (L), Moderate (M) and High (H).

The approach is based on the following rationale:

- Biological activity measured by lethality has sufficient common phenomena to produce valid comparisons. The Ames *Salmonella* assay measures gene revertants rather than toxicity, but its results can be grouped in a similar fashion.

- Each assay, regardless of the type of response measured, has a maximum applicable dose above which the test data are virtually impossible to interpret because of nonspecific responses of the test organisms to the gross quantities of substance added.

- A structure is needed for data formatting and analysis that can categorize toxicity and mutagenicity data from diverse sources into a series of similar comparative categories.

- The categories (nondetectable, low, moderate and high) are sufficiently broad to allow for normal variability and species differences, yet are narrow enough to provide data upon which decisions can be made.

- The data from Level 1 testing are directed towards ranking of streams from the sampling site for further studies and decisions on applicable control technology; they are not intended for making human-risk estimates.

To ensure uniform data recording and translation of raw data into the final summarized form, standard data recording and data transition forms have been developed. The forms for recording original data are discussed in the three separate sections of Reference 9 for health, aquatic ecological and terrestrial ecological effects tests. Data transition forms are used in sequence for data summary and analysis. The critical data values determined for each assay; MEC, EC_{50}, LD_{50} or LC_{50}; are recorded on critical data summary forms. Health effects test data are summarized using the Health Effects Critical Data Summary Form (Figure 6.1) while aquatic test data are summarized using the Aquatic Ecological Effects Critical Data Summary Form (Figure 6.2). A standard critical data summary form for the terrestrial effects bioassays is under development. Test materials are then ranked, using the critical data values reported in

FIGURE 6.1

HEALTH EFFECTS CRITICAL DATA SUMMARY FORM[a]

Contract No. ____________ Technical Directive or Project No. ____________ Site Sampled ____________

			RAM Cytotoxicity [EC50][c]				Rodent Toxicity	
Sample Identification	Ames Mutagenicity [MEC][b]	CHO Clonal Toxicity [EC_{50}][c]	Viability	Viability Index	ATP	ATP/Per 10^6 Cells	LD_{50}[d]	Toxic Signs[e]

[a] The assays, observed parameters and evaluation criteria are presented in IERL-RTP Procedures Manual; Level 1 Environmental Assessment Biological Tests,

[b] MEC: Minimum Effective Concentration - Lowest concentration for any tester strain giving a mutagenic response.

[c] EC_{50}: Effective concentration that reduces the observed parameter to 50 percent of the appropriate negative control.

[d] LD_{50}: The dose lethal to 50 percent of treated animals.

[e] Toxic signs are identified in a numbered list in the Level 1 manual. Only the number is reported here.

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FIGURE 6.2

AQUATIC ECOLOGICAL EFFECTS CRITICAL DATA SUMMARY FORM[a]

Contract No. ____________ **Technical Directive or Project No.** ____________ **Site Sampled** ____________

	Fresh Water				Marine			
	Fish		Daphnia	Algae	Fish		Mysid	Algae
Sample Identification	96-hr LC_{50}[b]	TS[c]	48-hr EC_{50}[d]	120-hr EC_{50}[d]	96-hr LC_{50}[b]	TS[c]	96-hr EC_{50}[d]	96-hr EC_{50}[d]

[a] The assays, observed parameters and evaluation criteria are presented in IERL-RTP Procedures Manual; Level 1 Environmental Assessment Biological Tests.

[b] LC_{50}: The concentration lethal to 50 percent of the treated animals.

[c] TS: Toxic signs are identified in a numbered list in the Level 1 manual. Only the number is reported here.

[d] EC_{50}: Effective concentration that reduces the observed parameter to 50 percent of the appropriate negative control.

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the summary forms as the basis for the evaluation. Evaluation criteria are presented in the Results and Data Interpretation section for each assay. The ranking of each sample in each test performed is then recorded in the Bioassay Summary Table (Figure 6.3).

6.2 DEGREE OF SENSITIVITY OF LEVEL 1 BIOASSAYS

An understanding of the results and interpretation of bioassay data is dependent on a knowledge of the level of sensitivity of the bioassays. The minimal levels of detection indicate the amount of toxicants that might exist undetected in a mixture.

The concept of a minimal detectable level is amenable to bioassays with dichotomous responses (+) or (-), such as the Ames *Salmonella* assay, but is not readily applied to bioassays with continuous responses, such as those measuring an LC_{50}. The limit of detection of an LC_{50} is dependent on the sample size and other test parameters.

A recent EPA report (73) discusses some of the calculated minimum detectable levels of pure chemicals in several Level 1 health effects bioassays.

FIGURE 6.3

BIOASSAY SUMMARY TABLE

Technical Directive or Project No. ______________________

Contract No. ______________________

	Health Effects Tests				Ecological Effects Tests									
					Aquatic						Terrestrial			
					Fresh Water			Marine						
Sample Identification	AMES Salmonella	RAM Cytotoxicity	CHO Cytotoxicity	Rodent Toxicity	Fish	Invertebrate	Algal	Fish	Invertebrate	Algal	Plant Stress Ethylene	Root Elongation	Insect Toxicity	Notes

ND = No Detectable Toxicity
L = Low Toxicity
M = Moderate Toxicity
H = High Toxicity

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CHAPTER 7

LEVEL 1 QUALITY CONTROL AND QUALITY ASSURANCE REQUIREMENTS

7.1 INTRODUCTION

If Level 1 assessments are to be used as a basis for decisions regarding further bioassay assessment, it will be necessary to ensure the quality of the test data. Quality control standardization and standardized data documentation will contribute significantly to ensurance of test quality and reproducibility. The documentation for test-quality verification consists of:

1. Detailed description of the work to be performed, individuals responsible for the study, sampling location, and approximate dates of the sampling and the analysis.
2. Standard Operating Procedures (SOPs) which outline the details of specific laboratory operations and quality control procedures (including positive and negative controls).
3. Complete set of raw data including name of the individual collecting the data and the date on which the data was recorded.
4. Report of the study which includes analysis and interpretation of all data.

All aspects of the protocol, raw data and report should be consistent; any deviations require justification and detailed explanation.

The procedures described above are consistent with the intent and the requirements of the FDA Good Laboratory Practice (GLP) Regulations (74).

7.1.1 General Quality Control Required For Level 1 Bioassay Performance

A separate set of documents outlining recommended quality control and quality assurance procedures has been prepared and are available as a guide for laboratories conducting Level 1 bioassays (9). These guides outline the basic steps involved in the Level 1 procedures and provide sample quality control recording forms for sample data collecting. The quality control/ quality assurance documents will be especially helpful to laboratories beginning to conduct Level 1 testing.

7.2 REQUIREMENTS FOR QUALITY ASSURANCE

7.2.1 Quality Assurance Samples

In order to ensure the quality of test results from biological laboratories involved in the environmental assessment program, audit samples have been made available, either as blind samples during analysis of assessment samples, or separately as coded unknown samples.

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Coded laboratory assessment samples have been prepared to submit to laboratories wishing to ensure Level 1 testing proficiency. The audit substances have the following characteristics:

1. Physical properties similar to those of natural samples, but defined compositions to ensure reproducible preparation.
2. Stability when stored under normal ambient conditions.
3. A full range of bioassay responses from no detectable toxicity (or mutagenicity) to high toxicity (or mutagenicity).
4. Prepared in sets with different levels of difficulty assigned to each set, permitting different levels of discrimination in the quality assurance or certification process.

At the present time several coded audit samples which have been developed with the characteristics described above are available through the Process Measurements Branch, Industrial Environmental Research Laboratory, U.S. EPA, Research Triangle Park, NC 27711. The results of the tests conducted by the requesting laboratory will be evaluated against the results established for each sample by a reference laboratory with an extensive data base for test results with the audit samples in the appropriate Level 1 bioassays. The reports submitted by the requesting laboratory will also be evaluated for compliance with Level 1 protocols and reporting requirements. Tables 7.1 and 7.2 provide examples of items reviewed in the auditing process in addition to the actual test data. When the audit is completed, a full report will be prepared by the auditing laboratory and submitted to the laboratory which conducted the Level 1 testing.

TABLE 7.1 AMES SALMONELLA/MICROSOME MUTAGENESIS ASSAY QUALITY ASSURANCE AUDIT

Study Design	Quality Control	Report
Cell Maintenance	Cell Maintenance	Results
Preparation of Strains	Preparation of Strains	Protocol
Culture Media	Culture Media	Tables
Metabolic Activation	Metabolic Activation	Graphs
Preparation of Test Sample	Preparation of Test Sample	Summary
Assay Conditions	Data Acceptance Criteria	Organization
Data Evaluation Criteria	Data Evaluation Criteria	

TABLE 7.2 CHO CLONAL TOXICITY ASSAY QUALITY ASSURANCE AUDIT

Study Design	Quality Control	Report
Cell Maintenance	Cell Maintenance	Results
Prep. of Test Cultures	Prep. of Test Cultures	Protocol
Prep. of Test Sample	Prep. of Test Sample	Tables
Treatment of Cells	Treatment of Cells	Graphs
Data Evaluation	Data Acceptance Criteria	Summary
	Data Evaluation Criteria	Organization

7.3 REQUIREMENTS FOR QUALITY CONTROL

In addition to audit samples, the quality control documents referenced in 7.1 are also designed to define the level of documentation required to comply with the proposed FDA GLP regulations. From time to time it will also be necessary to review final bioassay reports for consistency and completeness based on the suggestions in this manual and the three sections of the quality control manual (9).

CHAPTER 8

ENVIRONMENTAL ASSESSMENT BEYOND LEVEL 1

Level 1 environmental assessment should provide an accurate ranking of emissions from stationary sources with respect to their potential toxicity. Moreover, the ranking should ensure that the toxicity is from the emissions as released into the environment. Level 1 assessment should also generate information concerning rate of effluent discharge into the environment and environmental fate of the emission.

A composite of summarized bioassay and chemical analysis data will provide a measure of toxicity and the potential for damage to the environment. This concept is outlined in the scheme in Figure 8.1. Data from this scheme could be used in several ways.

1. It may be used to conclude that there are no detectable toxic emissions generated at the particular site and that there is no need for further testing.

2. The Level 1 chemical and bioassay results may be adequate to rank streams for further assessment and to provide sufficient guidance for control technology to individual process streams. In this case, Level 1 tests may be used to monitor the effectiveness of the control procedures over both long periods of time and varying process conditions.

3. The data may warrant initiation of a full Level 2 assessment. The results of the Level 2 studies will confirm Level 1 results and indicate if additional assessment or monitoring should be undertaken.

Therefore, it should be emphasized that a reasonable amount of flexibility can be introduced when using Level 1 analysis techniques for applications beyond Level 1 environmental assessment. The decision to go into Level 2 analysis should be developed as comprehensively as possible, as this level may require additional tests beyond those specified in this manual for the minimum Level 1 bioassay testing matrix.

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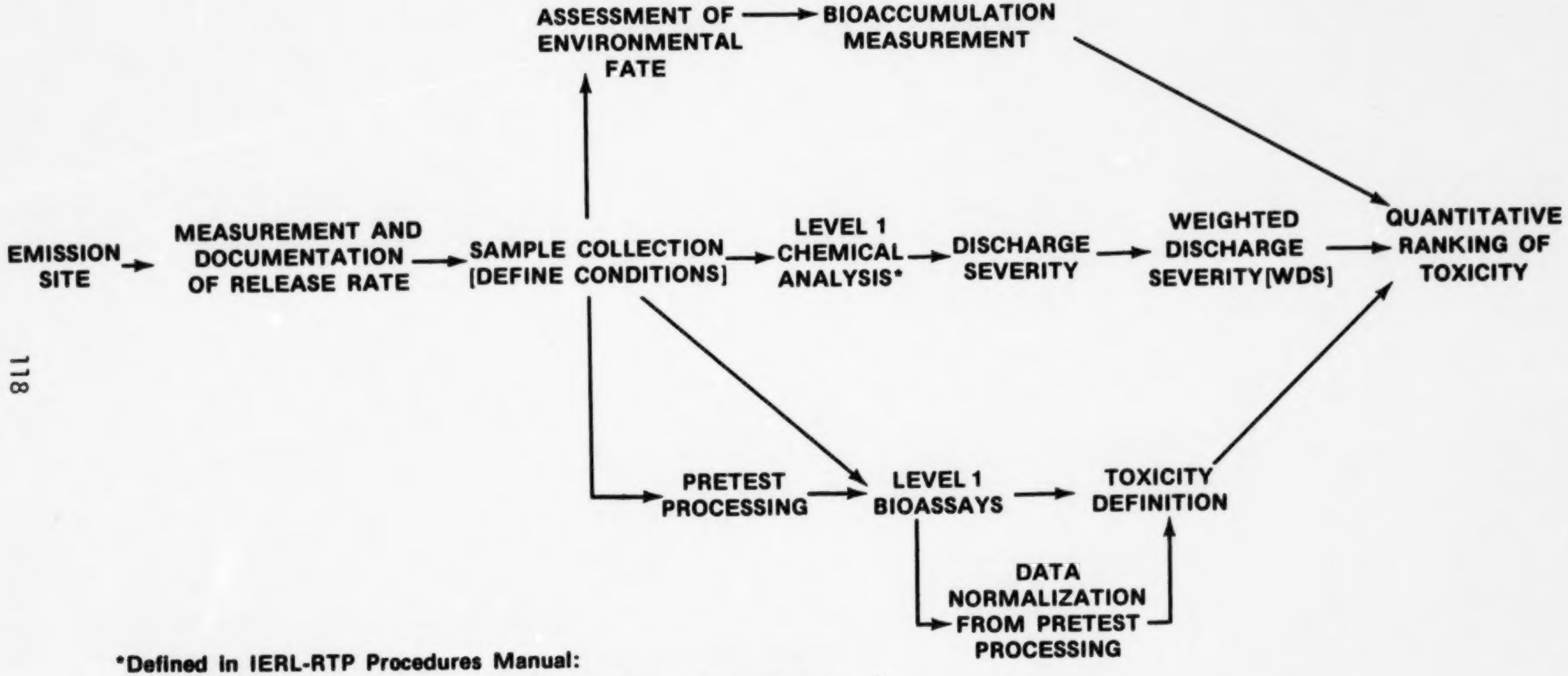
EMISSION SITE
MEASUREMENT AND DOCUMENTATION OF RELEASE RATE
SAMPLE COLLECTION [DEFINE CONDITIONS]
ASSESSMENT OF ENVIRONMENTAL FATE
BIOACCUMULATION MEASUREMENT
LEVEL 1 CHEMICAL ANALYSIS*
DISCHARGE SEVERITY
WEIGHTED DISCHARGE SEVERITY [WDS]
QUANTITATIVE RANKING OF TOXICITY
PRETEST PROCESSING
LEVEL 1 BIOASSAYS
TOXICITY DEFINITION
DATA NORMALIZATION FROM PRETEST PROCESSING

*Defined in IERL-RTP Procedures Manual:
Level 1 Environmental Assessment [Second Edition] [Reference 1]

Figure 8.1 PROPOSED SCHEME FOR A SECOND STAGE EVALUATION OF LEVEL 1 RESULTS.

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APPENDIX A

SISTER CHROMATID EXCHANGE IN CHINESE HAMSTER OVARY CELLS

A.1.1 INTRODUCTION AND RATIONALE

The objective of this *in vitro* assay is to evaluate the ability of a test sample to induce sister chromatid exchange (SCE) in Chinese hamster ovary (CHO) cells. This test is recommended to supplement the standard Level 1 health effects bioassays and is designed either to be run concurrently with the CHO clonal toxicity assay or, subsequently, after cytotoxicity information has been collected.

The frequency of sister chromatid exchanges is a very sensitive indicator of exposure of the genetic material in mammalian cells to chemical mutagens. The SCE test simply involves treating cultured cells with a test compound, growing the cells with 5-bromo-deoxyuridine (BrdU) for approximately 24 hours (two cell cycles) and making chromosome preparations that are stained for detection of SCE.

The chromosomes of dividing cells consist of two identical halves, or sister chromatids. To see exchanges between these (SCEs), a staining technique to differentiate between the chromatids is employed. This is achieved by growing cells in the presence of BrdU: after two cell cycles, one chromatid contains twice as much BrdU as the other and reacts differently to certain stains. Now one chromatid will stain intensely while its pair, or sister, is pale. Figure A.1 illustrates the formation of SCEs.

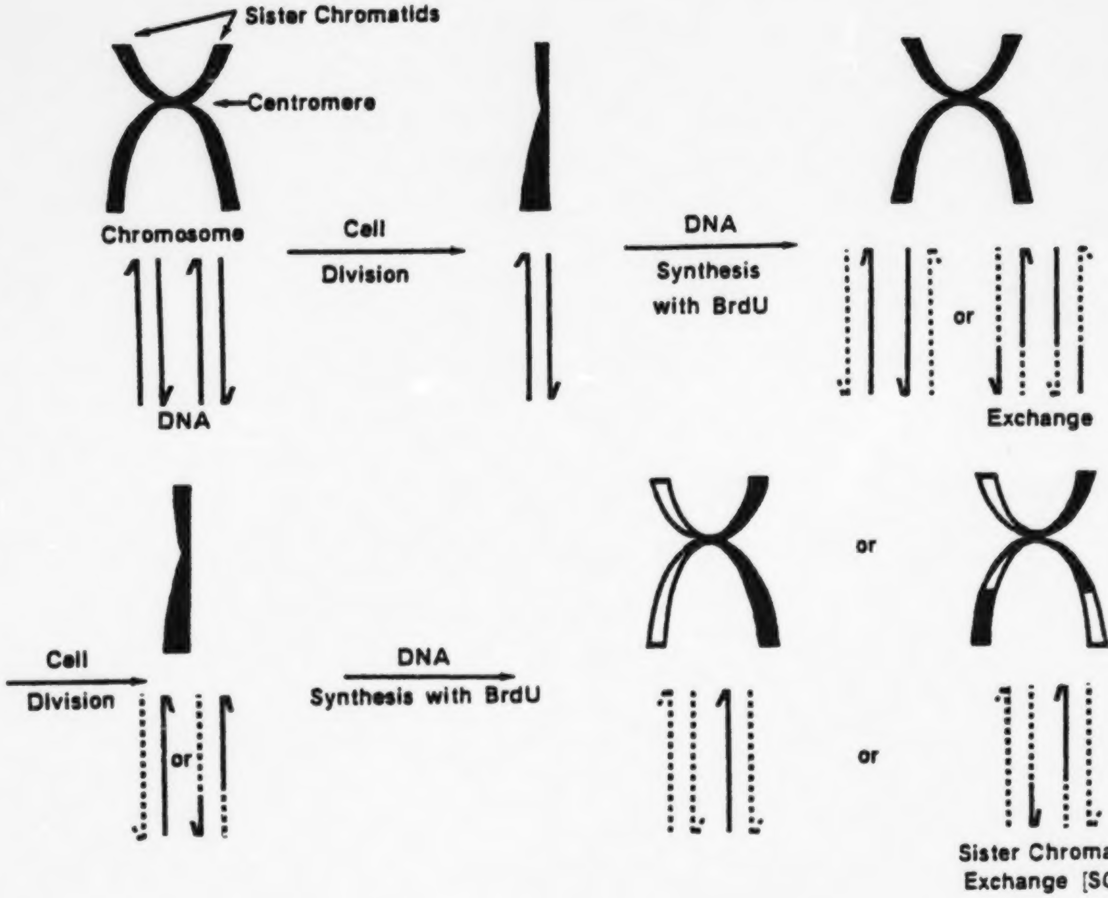
A.1.2 MATERIALS AND METHODS

Indicator cells. A cell line originally derived from Chinese hamster ovarian tissue and designated CHO-K1 is used for this assay. This cell type spontaneously transformed to a stable, hypodiploid line of rounded, fibroblastic cells with unlimited growth potential. Monolayer cultures have a fast doubling time of 11 to 14 hours and normally can be cloned at 80 percent or greater efficiency. Permanent stocks are maintained in liquid nitrogen and laboratory cultures are maintained by serial subpassage (not exceeding 15 passages). Laboratory cultures are periodically checked by culturing methods for the absence of mycoplasma contamination.

The cell line used is the same as in the rodent cell (CHO) clonal toxicity assay (Section 3.4).

Medium and cell cultures. CHO cells for this assay are grown in McCoy's 5a medium supplemented with 10-percent fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin. Cultures are set up approximately 24 hours prior to treatment by seeding 8×10^5 cells per 75 cm^2 plastic flask in 10 ml of fresh medium.

VISUALIZATION OF SISTER CHROMATID EXCHANGE [75]


Sister Chromatids
Centromere
Chromosome
DNA
Cell
Division
DNA
Synthesis
with BrdU
or
Exchange
or
Cell
Division
or
DNA
Synthesis with BrdU
or
Sister Chromatid
Exchange [SCE]

SCORING SCEs

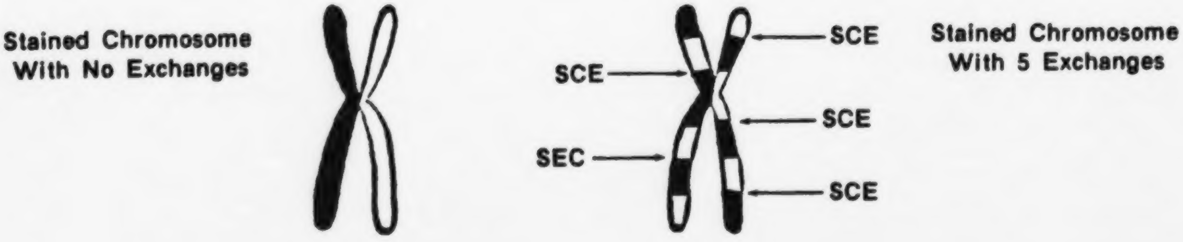

Stained Chromosome
With No Exchanges
SCE
SCE
SCE
SEC
SCE
Stained Chromosome
With 5 Exchanges

Figure A1 SISTER CHROMATID EXCHANGE [SCE]

Test material. This assay is compatible with test material of all sample forms except for nonparticulate-laden gases or vapors. Pretest sample processing will be as for the CHO clonal toxicity test, summarized in Table 2.4. If the CHO clonal toxicity test and the SCE test in CHO cells are run concurrently, the same stock dilutions of test material should be used for both experiments.

Immediately before use, a stock solution of the test compound is prepared in a suitable solvent such as culture medium, distilled water, dimethylsulfoxide, acetone or absolute ethanol. Serial dilutions are prepared in the same solvent to achieve desired final concentrations by addition of 0.1 ml of test solution to each 10 ml of culture, unless limited solubility requires use of a larger volume.

A.1.3 EXPERIMENTAL DESIGN

Dose selection. Cells will be tested by being exposed to the same range of concentrations of the test article as used in the CHO clonal toxicity test (Section 3.4). Selection of dose levels to process further and to score is based upon the concentration of test material which causes a reduction in the colony-forming ability of CHO cells by 50 percent (EC_{50}), as determined in the CHO test. Cells will be scored from the dose level closest to the EC_{50} and at two lower dose levels to include one non-toxic dose, if possible. If the EC_{50} is previously determined, doses selected to initiate the SCE test should include the EC_{50} concentration and four or five lower concentrations, ranging to non-toxic levels.

A second criterion for selection of the high dose for scoring is that a sufficient number of M2 cells (described below) is recovered to score.

Negative and solvent controls. The same sets of negative and solvent controls are ued as described in the CHO clonal toxicity test (Section 3.4).

Positive controls. A known direct-acting mutagenic and chromosome-breaking agent is used. Triethylenemelamine (TEM) is dissolved in water immediately before use and added to the culture medium. The final concentration is 0.025 μg/ml.

Cell treatment. In addition to the cells previously used for the CHO clonal toxicity test, approximately 3×10^6 cells are treated with the test article for 1 hour for the SCE test. Then, 5-bromodeoxyuridine (BrdU; 10 μM final concentration) is added to the culture tubes and incubation continued in the dark for 26 to 30 hours. If the test compound produces a marked amount of precipitate that would interfere with fixation and chromosome preparations, cells are washed with saline and fresh medium containing BrdU added, just prior to addition of colcemid. Colcemid is added for the last two to three hours of incubation (2×10^7M final concentration), and metaphase cells are collected by mitotic shake-off (76). The cells are swollen with 0.075 M KCl hypotonic solution, then washed three times in a fixative (methanol:acetic acid, 3:1), dropped onto slides and air-dried. Cell suspensions may be stored in fixative at 4°C until suitable dose levels for scoring have been selected.

Test for cell-cycle delay and repeated harvests. Because many compounds cause cell-cycle delay, a technique is used for assessing this and, if necessary, for performing later harvests on the same cultures. After two to three hours' incubation with colcemid, cells are harvested by mitotic shake-off and centrifuged to collect as a pellet. The supernatant medium can then be returned to appropriate flasks and reincubated at 37°C. After fixation of cells, test slides are made and stained for 10 minutes in Hoechst 33258* (0.5 μg/ml in phosphate buffer, pH 6.8), rinsed in water and mounted in the same buffer. These slides may be examined by fluorescence microscopy to assess the frequency of cells that have completed two cell cycles in BrdU. If there is significant delay, the same cultures may be harvested repeatedly until an adequate yield of cells showing complete differentiation between chromatids is obtained.

Staining and scoring of slides. Once dose levels for scoring are selected, slides are prepared and stained. Selection of dose levels for scoring is made (as described above) based upon the EC_{50} for clonal toxicity and the presence of sufficient M2 cells in each dose.

Staining for detection of SCE is accomplished by a modified fluorescence-plus-Giemsa (FPG) technique described by Perry and Wolff (77) and Goto (78). Slides are stained for 10 minutes with Hoechst 33258 (5 μg/ml) in phosphate buffer (pH 6.8), mounted in the same buffer and exposed at 55°-65°C to "black-light" from 15-Watt tubes for the amount of time required for differentiation between chromatids. Finally, slides are stained with 5-percent Giemsa for 10 to 20 minutes and air dried.

A.1.4 RESULTS AND DATA INTERPRETATION

M2 cells will be scored for the frequency of SCE per cell. Fifty cells will be scored per dose. Figure A.1 presents an example of scoring.

If an increase in SCE is observed, one of the following criteria must normally be met to assess the compound as positive:

> Two-fold increase: Approximately a doubling in SCE frequency over the "background" (solvent and negative control) levels, at a minimum of two doses.
>
> Dose response: A positive assessment may be made in the absence of a doubling if there is a statistically significant increase at a minimum of two doses and evidence for a positive dose response.

In some cases, statistically significant increases are observed with neither a doubling nor a dose response. These results are assessed according to repeatablity, magnitude of the response and proportion of the dose levels affected.

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*Supplied by Calbiochem-Behringer Corporation, LaJolla, CA 92037.

Statistical analysis employs a Wilkoxon Rank Sum Test (79) to compare SCE frequencies in treated cultures with negative and positive controls; the Jonkheere's test is used for a dose relation (79).

Evaluation criteria have not been developed at this point for ranking test samples based upon their ability to induce SCE in CHO cells. It will be sufficient to report a sample as positive or negative and the minimum concentration at which a positive response is observed.

GLOSSARY

Artemia: An aquatic arthropod, available dried, used as food for other aquatic organisms.

9AA: 9-aminoacridine; chemical used in the Ames assay as a positive control for strain TA-1537.

alveolar macrophage: Migratory, phagocytic cells located in the lungs which engulf and remove particulates, bacteria and foreign cells lodged in the lung.

Ames: Shortened name for Ames *Salmonella*/microsome mutagenesis assay; one of the Level 1 health effects tests.

ANTH: 2-anthramine; a mutagenic chemical used as a positive control in the Ames assay for all strains with activation.

Aroclor 1254: A polychlorinated biphenyl preparation used to induce liver enzymes in rats prior to the preparation of the S-9 fraction.

ATP: Adenosine triphosphate. The chemical in living cells that provides the primary source of energy. Cellular ATP levels are used in the RAM assays as an indicator of cell viability.

auxotrophy: The condition, under genetic control, in which a cell can not synthesize an essential nutrient; that nutrient must be supplied in the culture medium to allow growth.

base pair substitution: The substitution of a nucleotide base pair for the original base pair in double stranded DNA, which results in a change in the information content of the DNA.

bioaccumulation: The biological process by which organisms concentrate ambient chemicals in tissues and/or organs.

BrdU: 5-bromodeoxyuridine; a chemical analog of thymidine which the cell incorporates into DNA in place of thymidine.

caudal peduncle: In a fish, the base of the tail (caudal) fin (generally the area where the tail narrows).

CHO: Acronym for a cell line derived from Chinese hamster ovary tissue. CHO also used to identify the Level 1 cytotoxicity test which uses CHO cells.

chromosome: A complex unit of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and proteins that replicates during cell division and is usually constant in number in the cells of any one kind of plant or animal.

clonal toxicity: A measure of toxicity based on the reduction in the colony-forming ability of single cells exposed to a test material. This forms the basis for the CHO clonal toxicity assay.

Coulter Counter: Electronic particle counting and sizing device manufactured by Coulter Electronics, Inc., Hialeah, FL.

Cucumis sativus: One of the test organisms used in the RE test. The common name is cucumber.

Cyprinodon variegatus: Test organism used in the Level 1 marine aquatic ecological effects bioassay using fish. The common name is the sheepshead minnow.

Daphnia magna: Freshwater invertebrate used in the Level 1 freshwater aquatic ecological effects bioassay using macroinvertebrates. Common name for *Daphnia* is the water flea.

DMSO: Dimethylsulfoxide. A common laboratory solvent used to dissolve or suspend water-insoluble samples. DMSO, at low concentrations, is compatible with test organisms used in most Level 1 tests.

dose-response: The relationship between a biological response (an assay parameter) and the applied concentration of a test material.

Drosophila melanogaster: Insect used in the Level 1 Insect Toxicity Test as part of the terrestrial ecological effects bioassays. Common name for *Drosophila* is the fruit fly.

EC_{50}: Effective concentration; the estimated or calculated concentration of test material that causes a reduction in an observed parameter by 50 percent relative to the appropriate control.

EDTA: Ethylenedinitrilotetraacetic acid; a chelating agent.

EMEM: Eagle's Minimum Essential Medium with Earle's salts. The standard culture medium for the cultivation of rabbit alveolar macrophage cells.

ephippial eggs: Specialized eggs usually produced for overwintering. Ephippia are produced in response to adverse growth conditions.

eukaryotic: A cell type typical of higher plant and animal forms. Eukaryotic cells have specilized traits such as nuclear membrane and DNA organized into chromosomes.

FBS: Fetal bovine serum. A required growth supplement for culture media used in the RAM and CHO cytotoxicity assays.

femtogram: Unit of weight equal to 10^{-15} gram, used to measure ATP concentration in RAM cells.

foliar injury: Visable injury to the leaf surfaces of a plant.

FPG: Fluroescence-plus-Giemsa technique for staining cells for sister chromatid exchange.

frame-shift mutation: A quantitative change (addition or deletion) in the number of nucleotide pairs, in a DNA molecule, which alters the informational content of the DNA.

fugitive emission: Any emission transmitted to the environment without passing through some stack, duct, pipe or channel designed to direct or control their flow. The sample may be gaseous, particulate/aerosol or liquid.

gavage: To introduce material into the stomach by a tube.

Giemsa stain: A histological stain used for staining blood and other cells.

GLP: Good Laboratory Practices. A set of regulations developed by the U.S. FDA to define standards for nonclinical health effects studies.

GRAV: Acronym for the gravimetric method for determining the content of nonvolatile compounds in liquid sample. Determined by weighing the residue of a known volume of liquid.

hemocytometer: A glass chamber of precise volume, divided by grid lines into defined areas, used in conjunction with a microscope, to determine the number of cells per unit volume of fluid.

histidine: An amino acid ($C_6H_9N_3O_2$) essential to the growth of the strains of *Salmonella typhimurium* used for the Ames mutagenesis assay. The biochemical pathway for the biosynthesis of histidine is under genetic control and reversions to prototrophy for this pathway form the basis for detecting mutagens.

HPLC: High-performance liquid chromatography.

hypocotyl: Part of the stem below the cotyledons (primary leaves) in the embryo of a plant.

IERL-RTP: Industrial Environmental Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711.

incubation: The maintenance of living organisms (e.g., bacteria, mammalian cells in culture) in favorable conditions to promote growth.

instar: Developmental stages between molts in *Daphnia*, and other related animals.

IT: Insect toxicity test. One of the Level 1 terrestrial ecological effects bioassays.

in vitro: Outside the living body; tests or test conditions involving living cells maintained in an artificial (laboratory) environment.

in vivo: In the living body; tests or test conditions involving intact plants or animals.

Lactuca sativa L.: One of the test organisms used in the RE test. The common name is lettuce.

LC_{50}: Lethal concentration; the estimated or calculated concentration of a test material that is lethal to 50 percent of the test organisms during coutinuous exposure for a specified period of time.

LD_{50}: Lethal dose; the estimated or calculated dose of test material that is lethal to 50 percent of the test organisms following exposure to a single dose of test material.

M2 cells: Cells which have proceeded through a second cell division after a defined point such as following addition of 5-bromodeoxyuridine or addition of test material.

MAD: Maximum applicable dose. The highest concentration recommended for routine Level 1 testing for a given test.

manometer: A U-shaped instrument used for measuring air or gas pressure.

MEC: Minimum effective concentration. The lowest concentration giving a positive response according to the evaluation criteria in the Ames assay.

microsome: A cellular fraction consisting of membrane fragments and organelles that contain the enzymatic activities that biotransform chemicals. Liver cells are a good source of microsomes that contain enzymes associated with drug and chemical metabolism.

MOPS: Morpholinopropanesulfonic acid. The chemical used as a buffering system in the analysis of ATP content in rabbit alveolar macrophage cells.

mutation: Alteration of a heritable characteristic of a living organism usually resulting from a molecular change in the organism's deoxyribonucleic acid (DNA).

Mycoplasma: A genus of bacteria which do not contain a true cell wall. They are occasional contaminants of mammalian cells in culture.

Mysidopsis bahia: Marine invertebrate used in the Level 1 marine aquatic ecological effects bioassay using macroinvertebrates.

NF: 2-nitrofluorene; a mutagen used as a positive control in the Ames assay for strain TA-98 without activation.

NOEC: No observed effect concentration. The maximum concentration of test material which produces no observable deleterious effect by any criteria under study.

PBS: Phosphate buffered saline. A physiological saline used for procedures (such as washing) with mammalian cells in culture.

PCB: Polychlorinated biphenyl. See Arclor 1254.

phagocytosis: The process of ingestion and destruction of particulate matter, microorganisms or foreign cells by certain cells known as phagocytes. RAM cells are considered to be phagocytic.

Phaseolus vulgaris L.: Test organism used in the PSE test. The common name is the bush bean.

Pimephales promelas: Test organism used in the Level 1 fresh water aquatic ecological effects bioassay using fish. The common name is the fathead minnow.

plastochron index: Numerical index of the developmental status of plants derived from leaf length.

PMB: Process Measurements Branch. The branch in the Industrial Environmental Research Laboratory, U.S. EPA, Research Triangle Park, NC responsible for the development and validation of the Level 1 environmental assessment testing program.

POM: Polycyclic organic material.

prokaryotic: Cell type characteristic of simple organisms such as bacteria, viruses and some blue-green algae, in which the genetic material is arranged essentially into one chromosomal complex not separated by a membrane from the rest of the cell.

prototrophy: The condition, under genetic control, where a cell can synthesize an essential nutrient. The cell can grow in culture medium devoid of that essential nutrient.

PSE: Plant stress ethylene test; one of the Level 1 terrestrial ecological effects bioassays.

quantal test: In Level 1 *in vivo* rodent toxicity testing, the preliminary all-or-nothing test at the maximum applicable dose level to determine if a test material is toxic.

quantitative test: In Level 1 testing, the definitive portion of the *in vivo* rodent toxicity test using multiple dose levels to determine the LD_{50} of a test material.

radicle: The lower part of the axis of an embryo seeding which will form the root.

RAM: Rabbit alveolar macrophage cells or the Level 1 cytotoxicity test which uses these cells. See alveolar macrophage.

Raphanus sativus L.: One of the test organisms used in the RE test. The common name is radish.

RE: Root elongation test; one of the Level 1 terrestrial ecological effects bioassays.

reverse mutation: A mutation that restores the original genotypic (or phenotypic) condition of a cell that was lost by an initial mutation.

rotameter: A calibrated flow meter used to measure flow rate of a gas.

S9 mix: The liver homogenate preparation (S-9) derived from Aroclor 1254-induced Sprague Dawley rats combined with several cofactors to maintain hepatic enzyme activity. The term S-9 refers to the supernatant (which contains the microsomes) obtained after centrifuging the liver homogenate at 9000 x g. Used to biotransform test chemicals in the Ames assay to detect mutagenic activity.

Salmonella typhimurium: Bacterial test organism used in the Ames *Salmonella*/microsome mutagenesis assay. Four different histidine requiring strains are used in the Level 1 assay; TA-98, TA-100, TA-1535 and TA-1537.

SA: Sodium azide; a mutagenic chemical used as a positive control in the Ames assay for strains TA-1535, and TA-100 without activation.

SASS: Source Assessment Sampling System. Sampling system for sampling particulate laden gases developed by IERL-RTP and manufactured by Aerotherm Corporation, 485 Clyde Avenue, Mt. View, CA.

SC_{20}: Stimulatory concentration; the calculated concentration of a test material that causes a stimulation in growth of 20 percent relative to the appropriate control during continuous exposure for a specified period of time.

SCE: Sister chromatid exchange or the test which detects SCE. Microscopically visible exchange of portions of chromatid arms of the same chromosome. Elevated levels of SCE are indicative of damage to the genetic material.

Selenastrum capricornutum: Test organism used in the Level 1 freshwater aquatic ecological effects bioassay using algae. The organisms is a unicellular, non-motile chlorophyte.

Skeletonema costatum: Test organism used in the Level 1 marine aquatic ecological effects bioassay using algae.

SOP: Standard Operating Procedures. SOPs are explicit test procedures followed by personnel in a given laboratory.

TCO: Acronym for the total chromatographable organics method for determining the volatile organic content in a liquid sample. Determined by a chromatographic analysis of samples collected and stored under conditions that prevent volatilization of organics.

TEM: Triethylenemelamine; a direct-acting mutagenic and chromosome-breaking chemical used as a positive control in the SCE test.

Triticum aestivum L.: One of the test organisms used in the RE test. The common name is wheat.

trypan-blue: Biological strain used for diluting and staining cells for cell density and cell viability counting. Live cells exclude the dye and remain colorless while dead cells are stained blue.

trypsin: Chemical (enzyme) that digests extracellular proteins produced by mammalian cells that attach cells to one another and to substrates.

WAT: Acute *in vivo* toxicity test in rodents (whole animal test); one of the Level 1 health effects tests.

Trifolium pratense L.: One of the test organisms used in the RE test. The common name is red clover.

weanling mice: Mice that have recently been weaned. The age of weanling mice for the *in vivo* rodent toxicity assay is 21 to 28 days.

Wrights stain: A histologic stain developed for staining blood cells.

XAD-2: A porous sorbent resin used in sampling gaseous streams with the SASS train technique and in concentrating organic material from aqueous samples. This resin has a high affinity for non-polar species and a low affinity for polar species. XAD-2 is manufactured by Rohm and Haas Co., Philadelphia, PA.

XE-347: A porous sorbent resin used in concentrating organic material from aqueous samples. This resin has a high affinity for polar species. XE-347 is manufactured by Rohm and Haas Co., Philadelphia, PA.